Milk lipid complexation and interaction with food ingredients: Digestibility and absorption

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List of Publications

- **Publications**

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[2]. Zhiguang Huang, Letitia Stipkovits, Haotian Zheng, Luca Serventi, Charles S. Brennan (2019). Bovine Milk Fats and Their Replacers in Baked Goods: A review. Foods, 383(8), 1 – 20. http://doi:10.3390/foods8090383.

[3]. Zhiguang Huang, Hui Zhao, Wenqiang Guan Jianfu Liu, Charles Brennan, Maneesha S. Mohan, Letitia Stipkovits, Haotian Zheng, Don Kulasiri. Preparation and assessment of milk phospholipid-complexed antioxidant phytosomes: with vitamin C and E: A comparison with liposomes. Food chemistry, 2019 (submitted).

[4]. Zhiguang Huang, Hui Zhao, Wenqiang Guan Jianfu Liu, Charles Brennan, Maneesha S. Mohan, Letitia Stipkovits, Don Kulasiri. Milk phospholipids antioxidant activity and *in vitro* digestion: kinetics of fatty acids and choline releases. Journal of Functional Foods, 2019 (submitted.

[5]. Zhiguang Huang, Charles Brennan, Haotian Zheng, Maneesha S. Mohan, Letitia Stipkovits, Wenjun Liu, Don Kulasiri. The effects of fungal lipase-treated milk lipids on bread making. LWT – Food Science and Technology, 2019 (submitted).

[6]. Zhiguang Huang, Charles Brennan, Maneesha S. Mohan, Letitia Stipkovits, Haotian Zheng. Lipase-treated milk lipids on wheat, corn, and rice starch digestibility and functionalities: Amylose-milk fatty acid complex reduces glycaemic index. Journal of Agricultural Food Chemistry, 2019 (submitted).

[7]. Zhiguang Huang, Charles Brennan, Maneesha S. Mohan, Letitia Stipkovits. Milk lipid *in vitro* lipolysis kinetics in wheat, corn, and rice starch gels. International Journal of Food Science and Technology, 2020 (accepted).

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[9]. Zhiguang Huang, Charles Brennan, Maneesha S. Mohan, Letitia Stipkovitsa, Don Kulasiria. Milk lipid in vitro lipolysis kinetics in wheat, corn, and rice starch gels. The 16th ICC Cereal and Bread Congress (ICBC 2020, an oral presentation acceptance), Christchurch, New Zealand: 15 – 17 April 2020.

[10]. Zhiguang Huang, Charles Brennan, Maneesha S. Mohan, Don Kulasiri. Milk lipid effects on corn and wheat starch *in vitro* digestion (A poster presentation). The 2018 Global Food Science Student Competition, Food Science and Culture: Innovation, Communication and Collaboration. Jiangnan University, Wuxi, China: 14 – 18 November, 2018.

[11]. Zhiguang Huang, Charles Brennan, Haotian Zheng. The effect of dairy lipid and lipase on the properties of dough and wheat bread: An acceptance by The 3rd Food Structure and Functionality Forum Symposium and the 3rd IDF Symposium on Microstructure of Dairy Products. Montréal, Québec, Canada: July 2018

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[16]. Zhiguang Huang. Milk phospholipids and their complexing with food ingredients: Digestibility *in vitro* and functionalities (An oral presentation). 3-Min Thesis Competition in 2019 Post-graduate Conference of Lincoln University. Lincoln, Christchurch, New Zealand: August 8 – 9, 2019.

[17]. Zhiguang Huang, Charles Brennan, Maneesha S. Mohan, Letitia Stipkovits, Wenqiang Guan, Hui Zhao, Liu Jianfu. Milk phospholipids antioxidant activity and *in vitro* digestion: kinetics of fatty acids and choline releases (A poster presentation). 2019 Post-graduate Conference of Lincoln University. Lincoln, Christchurch, New Zealand: August 5 – 9, 2019.

[18]. Zhiguang Huang. Riddet 2019 Meeting – Submitted Thesis Video to Riddet Competition of Visualizing Your Thesis (VYT). Rotorua, New Zealand. September 30 – October 3, 2019.

[19]. Zhiguang Huang, Maneesha S. Mohan, Letitia Stipkovits, Don Kulasiri, Charles Brennan, Hui Zhao, Jianfu Liu, Wenqiang Guan. Preparation and assessment of milk phospholipid-complexed antioxidant phytosomes with ascorbic acid (A poster presentation). International Conference of Food Structures, Digestion and Health (FSDH). Rotorua, New Zealand. September 30 – October 3, 2019.

[20]. Zhiguang Huang, Maneesha S. Mohan, Letitia Stipkovits, Don Kulasiri, Charles Brennan, Hui Zhao, Jianfu Liu, Wenqiang Guan. Lipase-treated milk lipids on wheat, corn, and rice starch digestibility and functionalities (An oral presentation). The 2nd Asia – Pacific Grains Conference, ICC. Tianjin China: Nov 7 – 9 2019.

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Abstract of a thesis submitted in partial fulfilment of the
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Abstract

Milk lipid complexation and interaction with food ingredients: Digestibility and absorption

by

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Milk phospholipids and non-polar lipids can form conjugations with antioxidants and starches, in the matrices of phytosomes and milk lipid-starch complexes, respectively. In addition to complexation, milk lipids also interact with starches in food matrices, leading to physiochemical changes of associated foods. Milk lipids possess neutral colour, taste, odour, natural, clean-label, and non-allergic. However, there has been little research on interactions or complexes between milk lipids and other food components when milk lipid was used as an ingredients in different food matrices.

This thesis focuses on the extraction of bovine milk phospholipids from dairy products, purification of phospholipids, and preparation of vitamin C and E phytosomes in comparison with liposomes, followed by bovine milk phospholipid/triacylglycerol dispersions. Subsequently, non-polar lipids were used to prepare milk lipid-starch complexes in two kinds of food matrices: firstly bread and secondly starch gels. In the final research chapter, starch gel-stabilized milk fats were investigated in terms of lipid digestibility.

The phytosomes were made by food-grade ethanol evaporation, and were verified by FTIR, DSC, UV and CI. *In vitro* models were used to measure the phospholipid digestibility and cellular uptake. Amylose-lipid complexes were prepared by thermal methods. The complexes were then verified by spectroscopy analysis. Starch and milk fat digestibility was determined by *in vitro* assays and simulated by a multi-step reaction model.

The results on milk phospholipids showed that the polar heads of milk phospholipids in phytosomes interacted with hydroxyl groups of ascorbic acid resulting in the shifting of major bonds in the phosphatidyl residues in phospholipids, and therefore, milk phospholipid-based phytosomes had greater encapsulation efficiency and *in vitro* digestion stability than

liposomes. Additionally, in opposition to triacylglycerol, milk phospholipids showed greater lipid digestibility and exhibited antioxidant activity due to differences in molecular and hydrocolloid structure. Therefore, milk phospholipids have potential application in fortification of foods including infant formulas.

In contrast to milk phospholipid-based complexes, milk fats can interact and complex with corn, rice and wheat starches in food matrices. When milk fats were hydrolysed by fungal lipase, they formed starch-fatty acid complexes during baking, delaying bread firming rate and extending shelf-life due to reduced re-crystallisation of amylopectin. Hence, lipase treatment of milk fats offers a feasible way to improve both textural and physiological properties of bread. Also, milk fats-amylose conjugation can be produced by cooking at 95˚C, providing a practical method to lower the glycaemic response of starchy foods, reducing starch digestibility by 19% in corn starch, 17% in wheat starch, and 25% in rice starch.

In addition to conjugation with milk fats, starch particles can also be used to stabilize milk fat emulsions. *In vitro* digestion showed that the lipolysis reaction speed and extent of starch gel-stabilized milk fats were two – three-fold that of milk fat dispersion, confirming the calculation by the multi-step enzymatic reaction model. This indicates the possibility to regulate lipid digestibility by designing starch-based matrices.

Beginning with bovine buttermilk, membrane filtration was proposed to manufacture enriched milk phospholipid products (11 – 20 g polar lipids/100 g products), with the current, best available process efficiency. Supercritical fluid extraction was an effective, food-compatible method to produce high purity (65 – 90%) milk phospholipid products.

Overall, this thesis demonstrates the structure-property-functionality relationship of milk phospholipids/lipids in several food matrices. This thesis provided novel approaches to the further use of milk lipids as functional food ingredients, such as vesicles (Chapter 4), antioxidants for infant formulas (Chapter 5), bakery product textural improvers (Chapter 6), glycaemic index reducers of starchy foods (Chapter 7), and lipophilic compound carriers using starch-stabilized milk lipid matrices (Chapter 8). Last research chapter (Chapter 9) offered practical knowledge to develop new product and processes using milk phospholipids. In future research, besides milk phospholipid neuro-functionality, milk lipid interactions with proteins and phenolic compounds should provide interesting research questions to explore.

Keywords: Phospholipids, bovine milk lipid, phytosomes, complexing index, antioxidant activity, amylose-fatty acid complexes, lipolysis, lipase, emulsifier, shortening.

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List of Symbols and Abbreviations

%	Percent
°C	Degree of Celsius
µg	Microgram
µL	Microliter
AACC	American Association of Cereal Chemists
ABAP	2,2'-azobis (2-amidinopropane dihydrochloride)
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ACN	Acetonitrile
AMF	Anhydrous milk fat
ANOVA	Analysis of variance
AOAC	Association of Official Agricultural Chemists
AUC	Area under curve
BHT	Butylated hydroxytoluene
BI	Brownness index
BM	Buttermilk
BMC	Buttermilk concentrate
BMP	Buttermilk powder
BS	Beta serum
BSP	Beta-serum powder
C6	Hexane
CAA	Cellular antioxidant activity
CAD	Charged aerosol detector
CF	Carbon footprint
CI	Complexing index
CLSM	Confocal laser scanner microscopy
CM	Chloroform and methanol
CS	Corn starch
CSL	Calcium stearoyl-2-lactylate
CU	Cellular uptake
CV	Cell viability
CVD	Cardiovascular disease
DAG	Diacylglycerols
DATEM	Diacetyl tartaric acid ester of mono- and diacylglycerols
DCFH-DA	Dichlorodihydrofluorescein diacetate
DCM	Dichloromethane
DEE	Diethyl-ether
DF	Dia-filtration
DM	Dry matter
DME	Dimethyl ether
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNS	Dinitrosalicylic acid

DPPH	1,1-diphenyl-2-picrylhydrazyl (DPPH),
DSC	Differential scanning calorimetry
EDTA	Ethylenediamine tetraacetic acid
EE	Encapsulation efficiency
ELLS	The Euro-league for Life Sciences
ELSD	Evaporative light-scattering detector
ESI	Emulsion stability index
FAME	Fatty Acid Methyl Esters
FBS	Fetal bovine serum
FFA	Free fatty acids
FID	Flame ionization detector
FSDH	Food Structures, Digestion and Health
FTIR	Fourier transformation infrared
FV	Final viscosity
g	Gram
GC	Gas chromatograph
GF	Gel firmness
GGE	Glycaemic glucose equivalent
GI	Glycaemic index
GPO-PAP	Glycerine phosphate oxidase peroxidase
h	Hour
HACS	High amylose corn starch
HPLC	High-performance liquid chromatography
HPMC	Hydroxypropyl methylcellulose
HUJI	The Hebrew University of Jerusalem
ICC	The International Association for Cereal Chemists
IDF	The International Dairy Federation
IEC	Intestinal epithelial cells
IPA	Iso-propanol
LCA	Life-cycle assessment
LCFA	Long-chain fatty acids
LFL	Lipase-treated flour lipid
LML	Lipase-treated milk lipid
LPS	Lipopolysaccharide
LU	Lincoln University
M	Molar
MAG	Monoacylglycerols
MCFA	Middle-chain fatty acids
MDA	Malondialdehyde
MeOH	Methanol
MF	Microfiltration
MFA	Milk fatty acids
MFG	Milk fat globule
MFGM	milk fat globule membrane
mg	Milligram

min	Minute
mL	Millilitre
MPL	Milk phospholipids
MS	Mass spectroscopy
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MUFA	Mono-unsaturated fatty acids
NZIFST	New Zealand Institute of Food Science and Technology
OSI	Oxidative stability index
PA	Phosphatidic acid
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PE	Phosphatidylethanolamine
PFD	Process flow diagram
PG	Phosphatidylglycerol
PGEF	Polyglycerol esters of fatty acids
pH	Potential hydrogen
PI	Phosphatidylinositol
PLA2	Phospholipase A2
PLD	Phospholipase D
p-NMR	Pulsed nuclear magnetic resonance
Poly-60	Polysorbate-60
PRDMC	Phospholipid rich dairy milk concentrated
PS	Phosphatidylserine
PSD	Particle size distribution
PT	Pasting temperature
PUFA	Polyunsaturated fatty acids
PV	peak viscosity
Q-TOF-MS	Quadrupole time of flight mass spectrometry
RP	Reverse phase
RPE	Reverse phase evaporation
rpm	Rotations per minute
RS	Rice starch
RVA	Rapid viscosity analysis
SCFA	Short-chain fatty acids
SE	Sucrose esters
SEM	Scanning electron microscopy
SFA	Saturated fatty acids
SFE	Supercritical fluid extraction
SFI	Solid fat index
SIF	Simulated intestinal fluid
SM	Sphingomyelin
SPE	Solid phase extraction
SR	Scavenging rate
SSL	Sodium stearoyl lactylate

TA	Thermal aggregation
TAG	Triacylglycerol
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substance
TDF	Total dietary fibre
TFA	Trans-fatty acids
THF	Tetra hydrogen furan
TL	Total lipid
TLC	Thin layer chromatography
TMA	Trimethylamine
TPA	Texture profile analysis
TUC	Tianjin University of Commerce
UF/DF	Ultra-/dia-filtration
USDA	The United States Department of Agriculture
USP	US Pharmacopeial
UV	Ultra violet
VYT	Visualizing your thesis
w/v	Weight by volume
w/w	Weight by weight
WCS	Waxy corn starch
WI	Whiteness index
WPC	Whey protein concentrate
WPPC	Whey protein phospholipid concentrate
WS	Wheat starch

Chapter 1: Introduction

1.1 Background

In the last decades, a great deal of knowledge has been accumulated on the nutritional value and vesicle properties of milk fats and their tri-layered milk fat globule membrane (MFGM) structure. In 2005, it has been reported that MFGM acts as an emulsifier and nutrient delivery system with anti-microbial activity [1]. For instance, research has shown that MFGM doubles the bio-availability of vitamin E [2], improves ascorbic acid absorption via liposomes [3], enhances emulsification effects of milk lipids in food system [4], and functions as a bioactive component [5].

Phospholipids in bovine milk are emerging biomaterials with growing appeals in food and nutrition academics since the early 2000s. Thompson [6] first used milk phospholipids to prepare three kinds of liposomes. Since then, milk phospholipid-based liposomes have been prepared to encapsulate ascorbic acid [3] and lactoferrin by Liu *et al*. [7]. More recently, milk phospholipid liposomes were utilized to improve the storage stability of encapsulates [8], to enhance the encapsulate solubility and encapsulation efficiency [9, 10], to boost the bioavailability of encapsulate [11] and delivery efficiency [10]. Further, recent reports have summarized the health benefits of milk phospholipids including therapeutic aspects [12], infant's gut development and cognitive functions [13], and physiological functionalities [14]. Additionally, milk lipids and related dairy products, such as butter, anhydrous milk fat (AMF), cream, cultured milk fats, and cheese (matrix of milk lipids and proteins), have been incorporated into baked goods including bread, cakes, cookies, and biscuits, to improve the physiochemical properties of bakery foods [15].

There have been several reports on milk phospholipid-based liposomes recently. Thus, no previous research has investigated phytosomes produced out of milk phospholipids. In addition, lipases have been used to treat wheat flour lipids to produce endogenous emulsifiers, to improve bakery product quality. Nonetheless, there has been little research on using lipase-treated milk lipids in the bakery industry. Therefore, it is of interest to survey the interaction and complexation of milk polar- and non-polar lipids with starch and antioxidants in food systems.

1.2 Research gaps

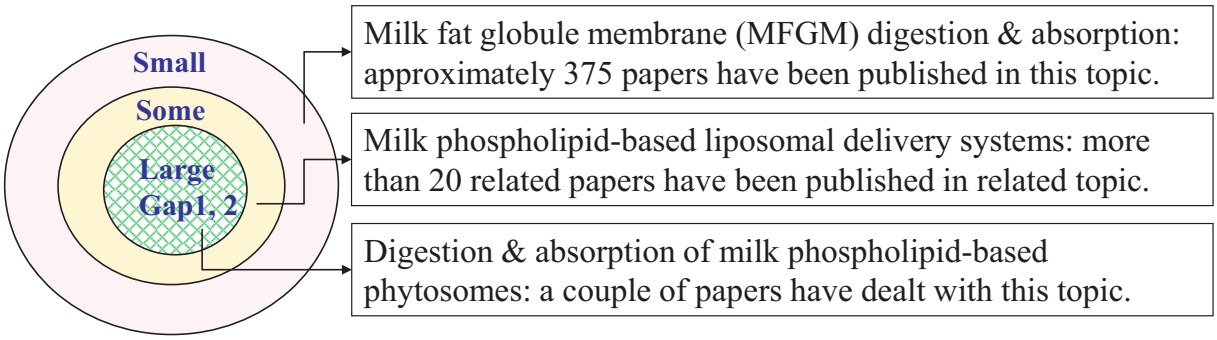
In the last decades, approximately 400 publications have been published, dealing with such aspects as MFGM structure, MFGM isolation and supplementation to food systems, phospholipid composition change with process and lactation, lipid-omics, MFGM digestibility and absorption, as listed in Table 1-1. Also, more than ten papers have been published, regarding liposomal delivery system using milk phospholipids. Hence, there are obvious research gaps in relation to milk phospholipid-derived complexes (*e.g.* phytosomes).

Similar to milk polar-lipid complexes, the research on lipid digestion and absorption in different food matrices has been gradually broadened, as illustrated in Figure 1-1. However, there has been little research on milk lipid-starch complexes and how their interactions affect the digestibility of milk lipids and associated starches.

Table 1-1: Five research gaps of thesis work.

RG	Research Gaps	Corresponding research chapters
Gap 1	Though milk phospholipids have been used to prepare delivery systems such as liposomes, phytosomes, an important phospholipid-bioactive conjugation, have not been made from milk lipids.	Chapter 4
Gap 2	Milk phospholipids have been highly-purified for functional foods such as infant formulas, but the digestibility and antioxidant activity of pure phospholipids have hardly been studied.	Chapter 5
Gap 3	Milk lipids have been frequently used as functional ingredients in bread making. Nonetheless, using lipase-treated milk lipids as bread improvers has not been explored.	Chapter 6
Gap 4	Milk lipids are major a source of lipid intake, and rice, wheat and corn are the main sources of starch in food. Milk lipid-starch complexes as a tool to lower glycaemic index of starches have been rarely studied.	Chapter 7
Gap 5	The outer layer of milk fat-encapsulated starch gel structure must be first disrupted to initiate lipolysis, therefore inner oil droplets may have a less easily digestible structure than untreated controls. Thus far, there have been no reports on how this starch-laden interface impacts on the internal lipid hydrolysis kinetics and the release kinetics of encapsulates inside the oil droplets.	Chapter 8
Gap 5	Thus far, no conclusion has been made on manufacture of milk phospholipids at large-scale. The	

- **Milk phospholipid-based phytosomes: digestion and absorption**



- **Milk fatty acid-starch complexes: digestion and absorption**

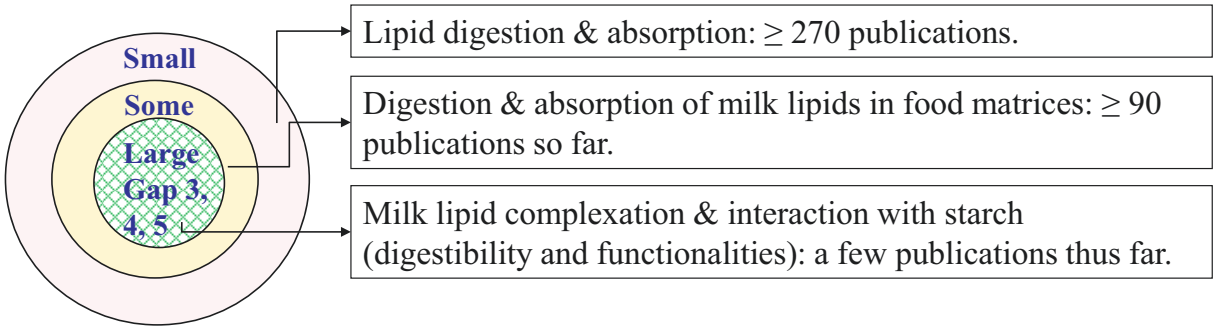


Figure 1-1: Visual of research gaps related to milk phospholipids or lipids complexes.

1.3 Aim of research

The overall target of this thesis was to study the structure-properties-functionalities relationships of bovine milk lipids in five food matrices: firstly, vitamin C and E phytosomes in comparison with liposomes (Chapter 4), followed by milk phospholipid/triacylglycerol dispersions (Chapter 5), subsequently milk fats in bread (Chapter 6) and then milk lipid-starch complexes (Chapter 7, finally starch gel-stabilized milk fats (Chapter 8). Chapter 9 assessed phospholipid-rich dairy streams and the available industrial production processes, using carbon footprint as a key parameter to compare each other.

To achieve this aim, we used bovine milk phospholipid to prepare phytosomal delivery systems (Chapter 4), to fortify dairy food nutritional properties (Chapter 5), facilitate milk lipid-based starch complexes to improve bread quality (Chapter 6) and enhance physiochemical properties starch in foods (Chapter 7), stabilize milk fat globules in starch gels for the purpose of regulation lipid digestibility and encapsulation lipophilic bioactive compounds (Chapter 8), and to implement industrial manufacture of milk phospholipids (Chapter 9), as specific goals listed in Table 1-2.

1.4 Specific objectives

Table 1-2: Five specific research objectives.

Goal	Specific objectives	Corresponding chapters
1	To prepare milk phospholipid-complexed antioxidant phytosomes with vitamin C and E and to assess phytosomal encapsulation capacity and efficiency, digestibility, and cellular uptakes.	Chapter 4
2	To extract milk phospholipids from dairy products and to evaluate their digestibility and antioxidant activity, in comparison to triacylglycerols.	Chapter 5
3	To hydrolyse milk fats and incorporate hydrolysates into a bread recipes as a dough strengtheners and textural improvers.	Chapter 6
4	To prepare milk fatty acid-starch complexes by using fungal lipase-treated milk lipids to manufacture cheap resistant starch.	Chapter 7
5	To encapsulate milk fats into starch gels to regulate the lipolysis reaction rate constant of milk lipids and to deliver lipophilic food ingredients in starch matrices.	Chapter 8
6	To assess three phospholipid-rich milk products, to compare available industrial processes, to investigate their process intensity, and to account for their carbon footprint.	Chapter 9

1.5 Hypotheses

Milk phospholipids have been extracted for functional food ingredients, and milk fat products have been used to produce various bakery products, such as bread, cookie, and cakes. Many other phospholipids from soy lecithin have been used for nutrient delivery. Hence, it can hypothesized that milk phospholipids can also function as a delivery vesicle for nutrients. In addition, milk lipids may also be a valuable source to manipulate the glycaemic load of cereal products, by forming complexes with starch particles. In regards to each specific goal, thesis hypotheses were made in Table 1-3:

Table 1-3: Five research hypotheses.

Goal	Hypotheses	Corresponding chapters
1	Milk phospholipids-antioxidant phytosomes are more efficient carrier systems than liposomes.	Chapter 4
2	Hydrolysis rate and extent of milk phospholipids are different from triacylglycerol.	Chapter 5
3	Milk lipids can fulfil similar roles to flour lipids when lipase is added to produce emulsifiers to strengthen bread dough and improve loaf volume.	Chapter 6
4	Milk lipid-starch inclusion complexes will lower the glycaemic load of starches and alter the rheological properties of starch gels as well.	Chapter 7

5	Milk lipid lipolysis kinetics will be altered when starch particles are used to stabilize the milk fat matrices.	Chapter 8
6	Carbon footprint will be a convenient key parameter index to compare available milk phospholipid manufacture processes.	Chapter 9

1.6 Thesis structure

This manuscript-orientated thesis consists of five research chapters (Chapter 4, Chapter 5, Chapter 6, Chapter 7, and Chapter 8) and two review papers in Chapter 2, with additional detailed schematic illustrations and method discription in Chapter 3. The first two research chapters (Chapter 4 and Chapter 5) on phytosomal digestiblity and milk phospholipid antioxidant activity were submitted to the journals ("Food chemistry" and "Journal of functional foods"). The subseqent three research chapters (Chapter 6, Chapter 7, and Chapter 8) focussed on milk lipid-starch interactions and complexes were submitted to "LWT-Food Science and Technology", "Journal of Agricultural and Food Chemistry", and "International Journal of Food Science and Technology (accepted)", respectively. The two reviews in Chapter 2 on phospholipid-vesicle properties and milk lipid functionalities in bakery products have been published in "Journal of Food Bioactives" and "Foods", respectively. Product/process development (Chapter 9) have been submitted to "Foods" journal. In addition to journal article submission, the contents of each chapter have been presented at related national and international food science conferences, as illustrated in Table 1-4.

The materials in this thesis were all derived from dairy lipids. Milk polar lipids were complexed with antioxidants (ascorbic acid and α-tocopherol) in Chapter 4, whereas milk lipid hydrolysate was conjugated with amylose to improve textural properties of bakery products (Chapter 6) or to make resistant starch (Chapter 7). Chapter 4 and Chapter 5 dealt with polar lipids, and Chapter 6, Chapter 7, and Chapter 8 were related to milk lipid-starch interactions, as illustrated in Table 1-4. The related milk lipids were in the form of either nutrient complexes (*i.e.* phytosomes in Chapter 4 and starch-lipid complexes in Chapter 6 and Chapter 7) or within starch gel matrices in Chapter 7 and Chapter 8, in which milk lipid-starch interactions were elaborated, respectively.

In vitro digestion and cellular uptake methods were used throughout this thesis. Digestibility of milk phospholipid-derived phytosomes and milk phospholipids were studied in Chapter 4 and Chapter 5, respectively. Digestibility studies of milk lipid-derived resistant starches were conducted in Chapter 6 and Chapter 7, and *in vitro* digestion of milk lipids in starch gels were performed in Chapter 8, and product and process development in Chapter 9.

Table 1-4: Thesis structure and publication related to each specific research goal.

Chapter	Title	Highlights	Publication
Chapter 1	Introductions	○ Aims of research, novelty, and hypotheses	[12, 16]
Chapter 2	Literature review	○ Phospholipid vesicle properties and milk lipid roles in cereal products	[1, 2]
Chapter 3	Materials and methods	○ Preparation of phytosomal complexes ○ Preparation of starch-lipid complexes	N.A.
Chapter 4	Preparation and assessment of milk phospholipid-complexed antioxidant phytosomes with vitamin C and E	○ Milk phospholipids phytosomes was prepared ○ Polar part of phospholipids bonded hydroxyl group of ascorbic acid ○ Phytosomes showed higher encapsulation efficiency than liposomes ○ Phytosomal and liposomal cellular uptake were different	[3] [19]
Chapter 5	Milk phospholipids antioxidant activity and digestibility: kinetics of fatty acids and choline release	○ Milk phospholipids extraction and purification ○ Assessment of polarity effects on lipid digestion ○ Antioxidant activity analysis ○ Comparison of phospholipids and triacylglycerol enzymatic lipolysis	[4]
Chapter 6	The effects of fungal lipase-treated milk lipids on bread making	○ Milk fats lipolysis using fungal lipase ○ Adding milk fat hydrolysates into bread recipes ○ Bread dough strengthening ○ Improvement of loaf volume and bread anti-staling performance	[5] [11, 14]
Chapter 7	Lipase-treated milk lipids on wheat, corn, and rice starch digestibility and functionalities: Amylose-milk fatty acid complex reduces glycaemic index	○ Preparation of resistant starches using milk fatty acids ○ Starch-lipid complexes verification ○ Prediction of the complexing indexes ○ Reduction of glycaemic load of starches	[6] [15, 20]
Chapter 8	Milk lipids *in vitro* digestibility in wheat, corn, and rice starch gels	○ Stabilisation of milk fat globules in starch gels ○ Measurement of milk fats-laden gel viscosity ○ Assay of milk fat lipolysis reaction rate constants ○ Modelling and simulation of lipolysis kinetics	[7] [13]

Chapter 9	Product and process development	○ Assessment of phospholipid-rich streams ○ Comparison of process efficiency ○ Calculation of carbon footprint	[8]
Chapter 10	Conclusion	○ Summary of the research findings ○ Conclusions of both milk phospholipid and lipid study in Chapter 5 to Chapter 8 ○ Technical relevance and significance of five research chapters (Chapter 5 – Chapter 8) ○ Prospective research topics in the future	N.A.

This thesis used milk lipids to make functional ingredients and to carry out roles, such as reduction of the glycaemic index of starch in foods and to improve the bioavailability of bioactive compounds by complexation-based vesicles. Overall, this thesis was focused on the structural-properties-functionality relationship, using both nutritional and technical properties of milk lipids.

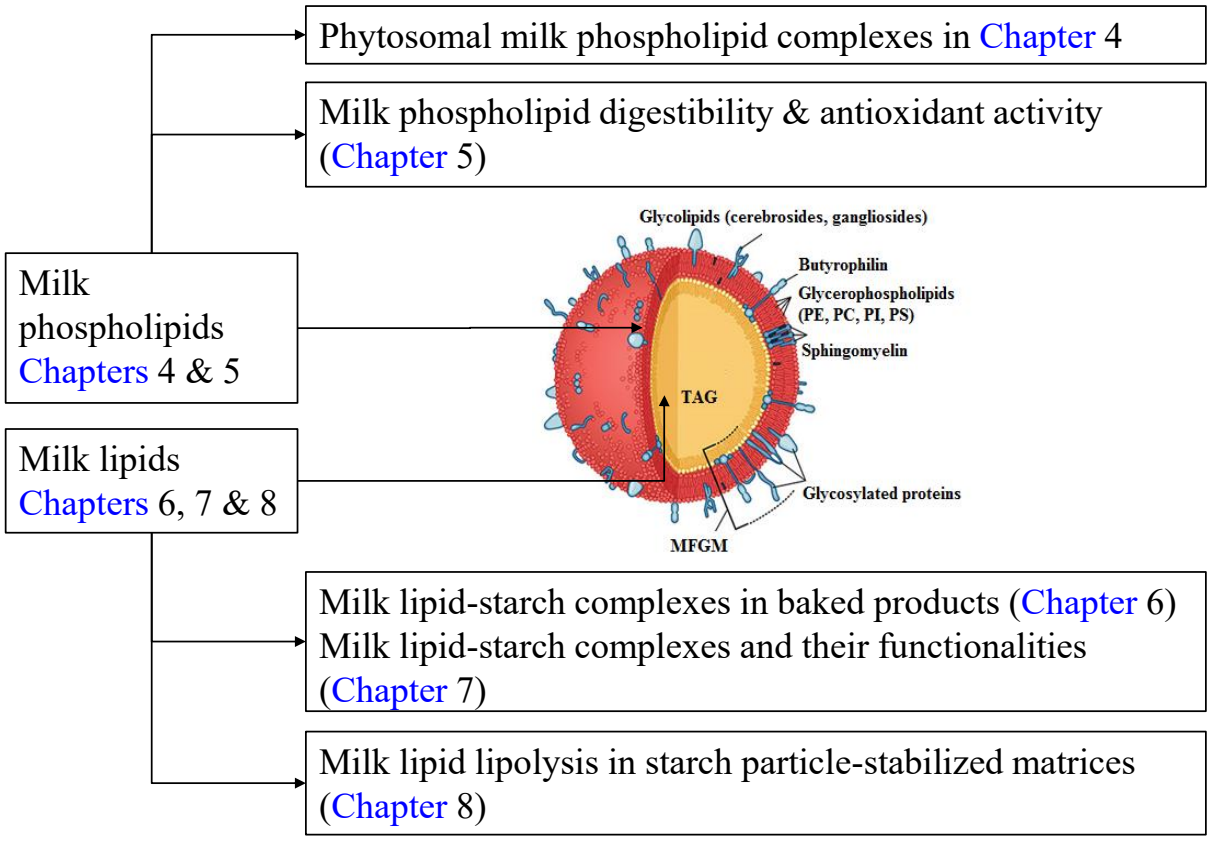


Figure 1-2: Schematic illustration of connections of each research chapter.

Chapter 2: Milk phospholipid vesicle properties and bakery milk lipids

Chapter 2 is a review of current related research on milk phospholipids and milk fats, and has been published in two food journals, respectively.

[1]. Huang, Z., Zhao, H., Guan, W., Liu, J., Brennan, C., Kulasiri, D., & Mohan, M. S. (2019). Vesicle properties and health benefits of milk phospholipids: a review. Journal of Food Bioactives, 5, 31 – 42. https://doi.org/10.31665/JFB.2019.5176.

[2]. Zhiguang Huang, Letitia Stipkovits, Haotian Zheng, Luca Serventi, Charles S. Brennan (2019). Bovine Milk Fats and Their Replacers in Baked Goods: A review. Foods, 383(8), 1 – 20. http://doi:10.3390/foods8090383.

Abstract: Phospholipids are important ingredients in milk. They serve as bioactive components with processing functionalities, despite representing only a small proportion of total milk lipids. There has been increasing interest in vesicle properties and health effects of milk phospholipids. However, there are limited reports on industry-scale manufacturing of related commercial products. This contribution aims to elucidate the industrial processes of manufacturing milk phospholipid products including phospholipid extraction and fraction as well as summarizing determination assays of milk phospholipids. In addition to industrial production, this review elaborates on application aspects, such as the biological properties of milk phospholipids and their technological importance as delivery vesicles of liposomes and phytosomes. In addition, new insights on large-scale production of milk phospholipids and new applications such as phytosomes and antioxidant properties are discussed.

Milk fats, and related dairy products, are multi-functional ingredients in bakeries. Bakeries are critical local industries in Western countries, and milk fats represent the most important dietary lipids in countries such as New Zealand. Milk fats perform many roles in bakery products, including dough strengthening, textural softeners, filling fats, coating lipids, laminating fats, and flavour improvers. This review reports on how milk fats interact with the ingredients of main bakery products. It also elaborates on recent studies on how to modulate the quality and digestibility of baked goods by designing a new type of fat mimetic, to make calorie- and saturated fat-reduced bakery products. It provides a quick reference for both retailers and industrial manufacturers of milk fat-based bakery products.

Keywords: Milk phospholipids; Solvent extraction; Liposome; Phytosome; Health effects; Vesicle properties; milk lipids; bakery products; fat replacer; shortening; baking activity

2.1 Introduction

2.1.1 Milk phospholipids as vesicles

Milk is a staple lipid source in the human diet [16]. Milk phospholipids have been used as materials for nutrient carriers since the early 2000s. Thompson [6] first used milk phospholipids to prepare three kinds of liposomes. Since then, milk phospholipid-based liposomes have been prepared to encapsulate ascorbic acid [3] and lactoferrin [7]. More recently, milk phospholipid liposomes were applied to improve the storage stability of encapsulates [8], the encapsulate solubility and encapsulation efficiency [9, 10] and the bioavailability of encapsulate [11], being more efficient than soy lecithin [10]. Furthermore, in terms of biological effects, several review papers have summarized various health benefits of milk phospholipids, with emphasis on therapeutic aspects [12], infant's gut development and cognitive functions [13], and physiological functionalities [14].

This contribution aims to summarize the industrial processes of manufacturing milk phospholipids, to update last five-year results on using phytosomes or liposomes to enhance bioavailability of bioactive compounds. It also reports on the recent trends on biological activities of milk phospholipids including antioxidant potential.

2.1.2 Milk lipids in bakery foods and their replacers

Milk plays critical parts in lipid intake of human beings [17]. Milk lipids consist of protein and also neutral lipids (tri-, mono-, di-acylglycerols (TAG, MAG, and DAG), free fatty acids (FFA)) and polar lipids (phospholipids) [18, 19]. Milk fats and related dairy products, such as butter, anhydrous milk fats (AMF), cream, cultured milk fats, and cheese (matrix of milk lipids and proteins), have been incorporated into both extruded and baked products, including breads, cakes and biscuits [20].

There are several reviews on bread lipids functionalities [20], bakery fat replacers [21], bakery lipids [22], lipid shortenings [23], bakery emulsifiers [24], bread functional ingredients and textural improvers [25], milk lipids in the food system [15], and bread emulsifiers [26]. Nonetheless, there has been no review on how milk fats perform their functions in bakery products thus far. Therefore, this review aims to summarize milk fat applications in the bakery industry, and to update results on using milk fats to enhance the quality and nutritional

value of baked goods. It also reports on the recent trends in relation to the health concerns of milk fats in baked products, and new ideas to reduce bakery energy density and saturated fatty acids (SFA).

2.2 Structure, composition and occurrence

2.2.1 Molecular structure

Milk phospholipids include two subclasses, glycerophospholipids and sphingolipids. Glycerophospholipids consist of a glycerol moiety with two fatty acids (lipophilic) esterified at positions of *sn*-1 and *sn*-2 and a hydroxyl group at *sn*-3 position, linked to a phosphate group and a hydrophilic residue. The structural details of the latter determine the types of glycerophospholipids, namely phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidyl-glycerol (PG), and phosphatidic acid (PA) [14]. The amphiphilic structure (lipophilic tail and hydrophilic head) provides milk phospholipids with emulsification properties.

Sphingolipids consists of a sphingosine backbone (2-amino-4-octadecene-1,3-diol), linked to a fatty acid through an amide bond and a polar head. Sphingomyelin (SM) is a prominent subclass of sphingolipids, having a phosphocholine head group. A minor constituent of sphingolipids in milk is glycosphingolipid, whose polar group comprises carbohydrate groups (glucose, galactcose, and lactose) [13].

2.2.2 Composition

Milk lipids represent approximately 4% of bovine milk [27]. Among total milk fat, only 0.32 – 1% represents phospholipid compounds [28]. Thus, it takes 2.5 – 8 litres of raw milk to produce one gram of phospholipids. Phospholipids are structured, functional lipids [29]. In all the three phospholipid sources, PC and PE contributes to the major proportion (52, 55 and 90% for milk, soy and egg yolk, respectively) of polar lipids. Compared with soy lecithin and egg yolk lecithin, milk phospholipids have a more balanced distribution in each subclass. SM and PS (24 and 12% in milk phospholipid profile, respectively), being regarded as functional ingredients for brain development [12, 30], are virtually absent in other sources, such as soy (0 and 0.5%, respectively) and egg yolk lecithin (1.5 and 0%, respectively) [31].

Apart from SM and PS profile, milk phospholipids have advantages over the other two sources due to their natural origin, oxidative stability and colour compatibility. Milk phospholipids have lower content of polyunsaturated fatty acids (PUFA 7.2 – 7.9% [32]) than

soy lecithin (60.37% [33]) and egg lecithin (23.2% [34]). Unsaturated fatty acids had a proportion of approximately 46.14% for mature bovine milk phospholipids [35], and 33 – 44.8% for two kinds of bovine milk polar lipids fed on maize silage and linseed [32], and whereas for the lecithin of soy and egg yolk, this percentage was 79.58 [33] and 54.6 [34], respectively. Thus, milk phospholipids are more resistant to oxidation than other phospholipids and they also have less colour intensity for this kind of fatty acid profile [36].

In terms of fatty acid profile of phospholipids, bovine milk, soy and egg yolk all have a predominant distribution of long chain fatty acids (LCFA 13 – 21), and the abundance of their LCFA is above 90% [34, 37]. The top two prominent fatty acids of phospholipids for milk and egg yolk are oleic and palmitic acids, which together account for more than 60%. The principal fatty acids of soy lecithin are linoleic and palmitic acids, contributing to 63.4 and 16.4%, respectively [38].

2.2.3 Occurrence

In intact raw bovine milk, phospholipids take the form of milk fat globule membrane (MFGM: 0.1 – 20 μm in diameter, 10 – 50 nm in thickness [39]). The triple-layer membrane consists of a surface-active inner monolayer enveloping triacylglycerols (TAG) in the centre, and an outer bilayer in contact with the aqueous phase of milk [12]. The milk fat globule membrane is composed of polar lipids, proteins, glycoproteins, enzymes and minor neutral lipids [40].

In dairy products, the triple-layer membrane structure becomes disrupted during processing and milk phospholipids redistribute into such products as buttermilk (BM) and β-serum powder (BSP, >60% lipid), which is an aqueous dairy stream through phase inversion from an oil-in-water to a water-in-oil emulsion [41].

2.3 Industrial manufacturing

2.3.1 Phospholipid extraction from dairy products

Milk phospholipid concentrated streams are related to butter processing, anhydrous milk fat (AMF) or whey fraction. Commercial milk phospholipid products are usually derived from dairy products, such as butter serum AMF, buttermilk, or acid butter whey. The level of phospholipids in these streams can be as high as 11.54, 2.03 and 1.84%, respectively [28]. Butter serum powder represents the highest level of phospholipid concentrate among those dairy streams. Therefore, it is a preferred feed for making milk phospholipid.

AMF, derived either from fresh cream or butter, contains purified milk fat (>99.8%) with removal of water and non-fat solid [27]. Butter serum AMF consists of highest proportion of phospholipids, with 11.54, 1.25 and 48.4% in terms of dry matter (DM), wet base and lipid base, respectively [42, 43]. Buttermilk, a product of churning process, is the serum of butter, containing the most of original milk whey proteins and less fat than butter [44]. Buttermilk phospholipids are less abundant than those of butter serum, with 2.03% of dry matter (DM) content. Acid buttermilk whey has a DM-based protein percentage of approximately 84.7%, containing 1.84 and 0.1% phospholipids for dry and wet products, respectively [42]. Intact milk fat globule membrane contains 30 – 70% polar lipids. However, it is generally only regarded as a laboratory source of phospholipids [45, 46]. Typical lipid composition of milk fat products is illustrated in Table 2-1.

Solvent extraction is one of the common methods to isolate milk phospholipids from dairy lipid concentrates. Ethanol is the most used solvent to extract milk lipids, for instance, hot alcohol (90%) extraction at 70˚C yields around 90% recovery rate [47]. Ethanolic extraction of lipids from proteins results in high purity (75%) phospholipids [48]. In a laboratory up-scaling test, supercritical carbon dioxide and 20% ethanol was utilized to extract lipids, and the final product had a purity of 56.24 ± 0.07% [49]. Supercritical carbon dioxide can only dissolve triacylglycerols without phospholipids, but together with near-critical dimethyl ether, both neutral and polar lipids are extracted [41]. Hexane is also a solvent that is occasionally used for lipid extraction [50]. Phospholipids are acetone-insoluble, but triacylglycerols dissolves in acetone. This selectivity of solubility also provides an approach to purify milk phospholipids [28, 35].

To obtain a high purity of phospholipids, lactose and protein (casein and whey protein) need to be isolated from lipids. Proteins can be denatured thermally or in acid solution (pH 4.6) [47, 51], the aggregated particles are then sieved by subsequent filtration. Starting with whey protein phospholipid concentrate, ethanol at 60 – 80 °C denatures proteins, resulting in phospholipid concentration of *ca.* 45.8% in the filtrates [47]. Proteolysis is also a viable way to remove proteins, in which whey and casein break into peptides and amino acids. Then the small molecules enter permeate after ultrafiltration (UF) or microfiltration (MF) operation [49, 52]. Alcalase (E.E. 3.4.21.62), a serine type endoprotease with esterase activity, catalysed amino esters at pH 7.5 and 35 – 60˚C [49], while tryptic and peptic hydrolysis may be carried out at 42˚C for 2 – 16 h, with pH at 7.7 and 2.0, respectively [52]. Lactose is a smaller molecule than lipid ant it also goes into permeate [53].

The process flow diagrams of industrial milk phospholipid manufacturing are not available due to commercial confidence. However, according to previous research reports, a block diagram was constructed to illustrate the principle of typical industrial production processes of milk phospholipids (Figure 2-1). Starting from butter serum or buttermilk, milk phospholipid concentrate can be refined by sequential unit operations of delactosing, deproteinising, and defatting [35, 36, 41].

Apart from the combined methods of solvents, filtration and centrifugation, milk phospholipids can be synthesized using lecithin phosphatidylcholine and milk L-serine (WO2005027822A2). First, the choline group of soy PC is cleaved with Phospholipase D, and replaced with an L-serine group in the presence of calcium salt. The synthesized PS 20/60 (21 and 62% PS, respectively) can acquire an unpleasant taste and may become undrinkable. Thus, oil capsules have been formulated to alter the flavour of PS. PS20/60 are physically unstable, and as they come from impure origin, these PS products (PS 20/60) were restricted by the public health authorities as described in WO2006128465A1.

Table 2-1: Phospholipid composition of three typical dairy products.

Product	PL on product (g/100g)	PL on DM (g/100g)	PL on fat (g/100g)	Protein on product (g/100g)	Protein on DM (g/100g)	Reference
Butter serum AMF	1.25	11.54	14.8 – 48.4	2.91	32.71	[42, 54]
Sweet buttermilk	0.16	2.03	4.49 – 33.1	3.31	32.95	[42]
Acid buttermilk whey	0.1	1.84	25.4	0.99	84.7	[43]

Notes: phospholipid (PL); dry matter (DM); anhydrous milk fats (AMF).

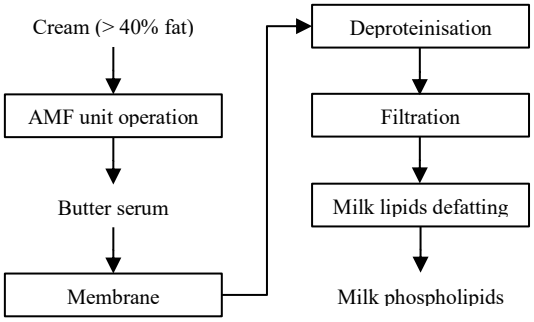


Figure 2-1: PFD to illustrate a typical routine of MPL isolation and purification.

2.3.2 Available commercial products and related patents

Among the milk phospholipid portfolio of Fonterra Co-operative Group Ltd, Phospholac 600 comprises of approximately 75% phospholipid, representing one of the most concentrated milk phospholipids in large-scale commercial products [31]. Its Phospholac 500/600/700 and Gangolac 600 have a phospholipid content of 34, 70, 62, and 15%, respectively [31, 55]. Additionally, Arla Foods amba have commercialized a series of phospholipid rich dairy milk concentrated (PRDMC) products, including Lacprodan® MFGM 10 and Lacprodan® PL 20/75. Lacprodan® MFGM 10 has been claimed to support physiological development of infant gut and provide infants with similar phospholipid benefits as breastfed infants because the fatty acid profile of Lacprodan® is similar to that of human milk [56]. In addition, PL 20 is made out of serum phase of butter oil product (AMF) with membrane filtration, yielding over 20% phospholipids in total solids, which is a pure-natural nutraceutical with properties that has not been discovered in conventional phospholipid sources including soy. PL 75 is a further ethanolic extract from PL 20, with 75% of phospholipids and protein-free. PL 20 and 75 targets infant milk formula and skin care, respectively [57].

As illustrated in Table 2-2, ethanol has frequently been used to extract milk lipids during industrial processes of manufacturing milk phospholipids. To further purify phospholipids, acetone (or dimethyl ether) is a common solvent to dissolve triacylglycerols. Most industrial milk products are generated from buttermilk (BM) and butter serum powder (BSP), except for some other origins such as whey protein concentrate (WPC) by Morinaga Milk Industrial Co Ltd. Tatua Co-operative Dairy Company produces a milk phospholipid concentrate from β-serum powder, an aqueous dairy ingredient separated from dairy streams comprising more than 60% lipids which has been made by phase inversion from an oil-in-water to a water-in-oil dispersion [58]. For instance, Lipidex, a derivative from β-serum powder by Synlait Milk Limited, contains 5 – 7% phospholipids and 26.6% fat in total [59]. Bovine milk SM (#860063, 25 – 200 mg) by Avanti has a purity of 99% [38]. Lecico Lipamine M20 comprises 20% of phospholipids including sphingomyelin, ceramides and ganglioside. This product has been produced with a special membrane technology, which used only water without using other solvents [60].

Table 2-2: Industrial manufacturing of milk phospholipids with 100 – 1000 kg input.

Patent	Assignee	Feed	Lipid extract	Defat	DM Purity (%)
US8471002B2-2013	Fonterra, NZ	BSP[a]	Ethanol, DME[c]	CO_2	25, 75, 60
US8231922B2-2012, WO2006128465A1	Arla, DK	BM whey	MF, ethanol		*Ca.* 19.1 (PS 2.3, SM 5.1)
US9814252-2017	Enzymotec Ltd, IL	Milk PL, egg yolk, *et al*	Ethanol and *n*-hexane	Acetone, CO_2	*Ca.* 24
US9700061B2-2017	Corman SA, BE	Cream	UF, DF[b]		20 – 39.4
JP2018052912A	Megmilk Snow Brand Co Ltd, JP	Milk PL, *et al*	Ethanol	Ion exchange resin or CO_2	*Ca.* 80
US5677472A-1997	Svenska Mejeriernas Riksforeni, SE	BM, whey	Ethanol, *n*-heptane	Acetone, heptane	*Ca.* 70
US9567356B2-2017	Cargill Inc, US	BM, lecithin	Alcohol (C1 – 3)	Acetone	55 – 75 PC
EP2168438A1-2010	Meggle JP Co Ltd	BSP[a]	High pressure ethanol	CO_2	*Ca.* 95
WO2002034062A1	Nv Marc Boone	Buttermilk *et al*	5 – 20 kDa UF[b]		*Ca.* 2.84
JP2005336230A	Morinaga Milk Ind Co Ltd	MFGM	Microfiltration	CO_2	*Ca.* 22
WO2007123424A1	Owen John Catchpole	Tissue *et al*	Alcohol (C1 – 3)	CO_2	5-79%

Notes: [a]Butter serum powder, buttermilk; [b]Ultrafiltration and dia-filtration; [c]Dimethyl ether.

2.3.3 Analysis: Sample preparation, fractionation and chromatography

For analysis proposes, milk lipid samples are usually prepared with solvent extraction. The Folch and Bligh extraction method using both chloroform and methanol, is a common formula to dissolve milk lipids. Though dichloromethane (DCM, less toxic than chloroform) has recently been introduced to replace chloroform [61], the principal methods of lipid extraction remain to be the Folch extraction [62], the Bligh method (chloroform/methanol/water, 1:2:0.8, v/v/v) [63], or the Röse-Gottlieb extraction of ammoniacal ethanolic solution of milk samples with diethyl ether and light petroleum [51, 64]. Total lipids of samples may be measured using gravimetric determination, Gerber-van Gulik butyrometer, infrared spectral method specified in International Dairy Federation (IDF) [51], or gas chromatograph equipped with a flame ionization detector (FID) [65].

Milk phospholipid fractions are usually further purified by a solid-phase extraction (SPE) before a determination assay of phospholipids and their subclasses, as illustrated in Table 2-3. Silica gel bonded cartridge is the most used SPE column to fractionate phospholipids from neutral lipids. First, the column is conditioned with hexane, then it is eluted by a mixture of

hexane (C6) and diethyl-ether (DEE) to separate triacylglycerols. After that, another elution with chloroform, methanol and water will recover the phospholipids from the SPE column, which will be collected for solvent evaporation by using rotary evaporation. The final product (phospholipids) after solvent drying is stored at -20˚C before using [66]. In addition, chloroform and methanol have been used as SPE conditioning and elution solvents [67]. Some SPE was performed with silica gel plate instead of silica gel bonded cartridges [35]. Total phospholipids in milk samples can be determined by IDF molybdate assay [68], Fourier transform infrared (FTIR) spectroscopy [69] or enzymatic method measuring the choline content [70].

Nuclear magnetic resonance (NMR) using 31P is a standard assay to quantify milk phospholipids and their subclasses [71, 72]. However, chromatography is the more common assay to determine milk phospholipids. Thin layer chromatography (TLC) is a convenient assay without sophisticated instruments. A formula of TLC elution solvent mixture containing hexane, diethyl ether and acetic acid (80:20:1, v/v/v) has often been applied on a silica gel plate. The fractionated subclasses are then visualized on the plate with iodine vapour [35, 73].

High-performance liquid chromatography (HPLC) remains the most commonly used method, because it can more accurately quantify the total phospholipids and each of their subclasses than TLC. For each HPLC assay, 5 – 10 µL sample (approximately 5 – 100 µg/mL) is the necessary amount to perform chromatographic analysis [63]. As shown in Table 2-3, HPLC has been usually coupled with such detectors as ultraviolet (UV) absorbance, evaporative light-scattering detector (ELSD) and mass spectroscopy (MS). Due to the polarity of milk phospholipid, silica column has often been used to separate the subclasses of milk phospholipids. To further fractionate the species of specified milk phospholipid subclasses, reverse phase (RP) HPLC with a C18 column can be employed [74]. The binary solvents of chloroform and methanol or acetonitrile and ammonium acetate are frequently used as an elution medium. The change of formula of elution solvents leads to the different detection order of phospholipid subclasses in the chromatogram, as illustrate in Table 2-3. In some cases, pH of mobile phase was modulated by trimethylamine or ammonia hydroxide (pH 3 and 6, respectively) and formic acid has shown benefits in providing a flat baseline [75].

Table 2-3: HPLC assays to determine milk phospholipids.

Milk sample origin	Lipid extract	PL fractionation	Stationary phase	Column size (L×D mm)	Particle size (µm)	Mobile phase	Isocratic or gradient (G) elution	Volume (mL/min)	Elution time (min)	Column oven temperature (◦C)	Detector	Identified species in order	Reference
Bovine raw	CM		Silica	100×2.1	1.7	2 ACN[f]	G	0.3	28	45	Q-TOF-MS[a]	PS, PG, PI, PE, PC, SM, LPC[d]	[76]
Cow, ewe, goat skim	DMC		Silica	500×4.5	5	4 CM[g]	G	0.5 – 1.4	53	35	ELSD	PE, PI, PS, PC, SM	[77]
Cow raw	CM		C18	100×2.1	1.7	2 ACN	G	0.225	21		Q-TOF-MS	PC, SM, LPC	[63]
Cow BMUF	CM		Silica	150×3	3	2 CM	G	0.5	20	35	ELSD-LTII[b] 50◦C 3.5Bar	PE, PC, SM, PS, PI	[51, 75]
Donkey raw	CM		Silica	150×3	3	2 AA MeOH DCM[h]	G	0.5	17		RP LC-MS	PI, PE, PS, PC, SM	[78]
Cow raw	CM	SPE (CM)	Betasil DIOL	150×4.6	5	3 C6 IPA FA[i]	G	1.5	19	30	CAD[c] 2.4 Bar	PI, PE, PS, PC, SM, LPC	[79]
Cow raw	CM	SPE (C6, DEE)[k]	Silica	250×4.6	5	2 CM	G	1	40		ELSD (3.1Bar 50 ◦C)	PE, PI, PS, PC, SM	[80]
Cow, yak raw	CM	SPE	Silica	8×3 250×3	5	2 ACN	G	1	24	50	ELSD (90 ◦C)	PI, PE, PS, PC, SM	[81]
Cow raw	CM		Silica	250×4.6	5	2 ACN	G	0.6	25	30	MS	PS, SM, PE, PC, PI, LacCer[e]	[82, 83]
Cow raw	CM		Silica	150×3	3	2 CM	G	0.5	35	40	ELSD (85 ◦C)	PE, PI, PS, PC, SM	[38]
Cow raw	CM		C18	32×0.1 200×0.1	3	2 ACN	G	0.5	200		MS	PI, PE, PS, PC, SM	[46]
HBM[j]	CM		Hypersil APS-2, aminopropyl	150×2.1	3	2 CM	G	0.25	29	25	MS	PI, PE, PC, PS, SM	[84]
Cow raw	CM		Silica	250×4.5	5	2 CM	G	1 – 1.5	60	40	ELSD	PE, PI, PS, PC, SM	[65]
Cow raw	CM	SPE (C6, DEE)	Silica	250×4.6	5	2 CM	G	1	40		ELSD 50 ◦C 3.2Bar	PE, PI, PS, PC, SM	[66]
Cow raw	CM	TLC (C6, DEE)	Silica	150×2.1	3	2 CM	G	1	55	40	ELSD	PE, PI, PS, PC, SM	[35]
HBM	CM	TLC (C6, DEE)	Silica	250×4.6	5	3 MeOH ACN[l]	I	1		30	UV205nm	PE, PC, SM	[85]
Cow	CM Röse		Silica	150×3	3	2 DCM MeOH	G		26	40	CAD 2.4Bar	PA, PI, PE, PS, PC, SM	[64]
Cow whey	CM		Silica	150×4.6	3	2 CM	G	0.5	27		ELSD 40 ◦C 1.8Bar	PE, PI, PS, PC, SM	[86]

[a]Quadrupole time of flight mass spectrometry; [b]evaporative light-scattering detector, low temperature; [c]charged aerosol detector; [d]lysophosphatidylcholine; [e]lactosylceramide; [f]acetonitrile and ammonium acetate; [g]chloroform and methanol; [h]acetic acid, methanol; [i]hexane, iso-propanol, di-chloromethane, formic acid; [j]human being milk; [k]hexane, diethyl ether; [l]methanol, phosphoric acid, and acetonitrile (32, 0.6, 67.4% in volume, respectively).

2.4 Vesicle properties

2.4.1 Liposomes

Milk phospholipid concentrate has sufficient emulsification properties due to its amphiphilic molecular structure. Milk phospholipids can also be used to deliver nutraceuticals and bioactive compounds in food and bio-pharmaceutical industries, increasing stability, solubility and bioavailability of the encapsulate [87]. In a recent report, the vesicle properties of milk phospholipids was thoroughly reviewed [39].

Milk phospholipid-based liposomes have been proven to deliver lipophilic or hydrophilic components to improve the bioavailability of encapsulates, in either pharmaceutical or food industries. In the cosmetic area, liposomes have been used to facilitate dermal absorption of active compounds. Milk phospholipid-based liposomes have been applied to co-deliver β-carotene within the membrane and ascorbic acid in the inner phase [88]. The complexing index increased to 26 ± 0.5% from *ca.* 14% when the milk phospholipid concentration was improved from 5 to 10%, then plateaued at 26 ± 0.5% when the milk phospholipid concentration was 10 – 15%. The size of carriers was 120 ± 2 nm using micro-fluidization unit. The samples aggregated during storage at pH 3, whereas the colloid remained stable after 7 weeks storage at pH 7 [3]. The liposome carriers based on milk phospholipids are shown in Table 2-4.

2.4.2 Phytosomes

As illustrated in Table 2-4, the phytosome carrier can also deliver bioactive compounds, both lipophilic and hydrophilic, to enhance oral bioavailability [89]. Phytosomes are physically-stable complexes, with a simple manufacturing process [90]. Complexing reaction of milk phospholipids and encapsulate (molecular ratio 1 – 5) was realized in either ethanolic or methanolic solution of 55°C. As a result phytosomal conjugation, the bioavailability of encapsulate was enhanced by 3 – 5-fold y compared with crude samples [91, 92], and whereas, the solubility of 36-fold increase was evidenced [93].

Both milk phytosomes and liposomes are derived from milk phospholipids. Liposomes encapsulate bioactive compounds in either the core of phospholipid globule or in the phospholipid bilayer, whereas phytosomes are different from liposomes because phospholipids conjugate with encapsulates. Therefore, they are more durable and efficient than liposomes [94]. Currently, milk phospholipid-based phytosomes are not yet explored, and it should provide a prospective area to study.

2.4.3 Gastrointestinal digestion and absorption of phospholipids

Milk phospholipids do not hydrolyse in the mouth and gastric tract, thereby they can be carriers of bioactive compounds [12]. Their digestion occurs in the lumen, the upper part of intestinal gut. Phospholipase A, B, C or D acts on either *sn*-1 or 2 acyl (A), both *sn*-1 and 2 acryl (B), *sn*-3 phosphoric base (C) and *sn*-3 polar head (D), respectively [95]). In human being, pancreatic phospholipase A2 (EC 3.1.1.4 [96]) can act upon *sn*-2 position of phospholipids, resulting in lysophospholipids and fatty acids. The fatty acid group of lysphopholipids can be further cleaved by lysophospholiase (EC 3.1.1.5) [97]. Furthermore, the pancreatic lysophospholipase of human beings is most likely a non-specific phospholipase, but carboxyl ester hydrolase (EC 3.1.1.1) [98]. In addition, sphingomyelinase (alk-SMase, EC 3.1.4.12) acts on phosphoric di-ester bond of sphingomyelin, generating ceramide and phosphocholine [99]. Ceramide will be further split by mucosal ceramidase (N-CDase EC 3.5.1.23) [100]. The lipolysis products then cross the border of epithelial cells (mucosa) and enter the enterocyte to synthesize new phospholipids, which are then incorporated into chylomicrons (CM). After that, approximately five hours postprandial, CM will enter into the lymph and blood circulation. Apart from absorption of hydrolysate of phospholipids (lyso-PLs and fatty acids), approximately 20% of phospholipids are passively absorbed in the intestinal lumen [12]. In addition, indigenous phospholipid excretion into bile is 10 – 20 g per day [101], which was much higher than endogenous phospholipids (2 – 8 g phospholipid ingestion per day) [102].

Table 2-4: Milk phytosomes and liposomes as bioactive compound carriers.

Encapsulate	Phospholipid	Vesicle	Bioavailability	Reference
Celastrol (CST)	Soy PC	Phytosomes	4 – 5-fold increase	[92]
Apigenin	Soy PC	Phytosomes	Up to 82%	[93]
Berberine (BER)	Soy PC	Phytosomes	3-fold increase	[91]
18β-glycyrrhetinic acid	Soy lecithin	Phytosomes	Extended storage to 30 – 90 days	[103]
Curcumin	Milk PL	Liposomes: Sonication	More efficient and stable than soy lecithin	[9]
Lactoferrin (LF)	Milk PL	Liposomes: Ethanol injection	Gastric stable and slow intestinal hydrolysis	[7]
Tea phenolic	Milk PL	Liposomes: Micro-fluidization	More efficient than soy lecithin	[8]
β-carotene and ascorbic acid	Milk PL	Liposomes Micro-fluidization	Poor physical stability upon storage	[88]
Silybin	Milk PL	Reverse phase evaporation (RPE)	10-fold increase	[104]

2.5 Health impacts

The nutraceutical value of milk polar lipids has been reviewed, including the efficacy for modification of the trajectory recession of cerebral structure in old age [105], the roles in the growth of infant brain and gut [13], the effects of immune-mediated anti-carcinogenic effects and anti-inflammatory activity [14], and the relevance to hepatoprotection and cardiovascular diseases [12]. Furthermore, milk phospholipids have been shown to reduce the waist circumference of the participants, compared with soy lecithin in a clinical trial, although the blood lipid concentrations of the attendants in the trial was not altered [106]. In addition, the effects of Lacprodan® PL-20 in supporting infant intestinal maturation [107] and a healthy microbiota [108] have been demonstrated clinically. Research has also illustrated that buttermilk and krill oil phospholipids may be associated with the improvement of synaptic signalling in aged rats [109].

2.5.1 Neurocognitive effects

The nutritional value of milk polar lipids includes gut development (SM), neurocognitive development (SM), liver protection (PC), bacteria inhabitation (lyso-phospholipids), maintaining homeostasis (PE), cell signalling (PI) and memory restoration (PS), as reviewed in previous reports [28, 110, 111]. It has been documented that milk phospholipids can enhance neurocognitive development. For example, research has shown that sphingolipid supplementation improved the myelination of central nervous system and was responsible for

the normal brain weight of rat infants [12]. L-serine is essential for the synthesis of sphingolipids and phosphatidylserine (PS) in particular types of central nervous system neurons [112]. Additionally, the cognitive performance benefits of dietary milk phospholipid have been evidenced with the clinical trial [113], the rats model [114], and the piglet model as well [115].

Table 2-5 illustrates the present results appertaining the cognitive functions of milk phospholipids, from either *ex vivo* models or *in vivo* models. Most tests conferred the benefits of milk phospholipids on brain function, however one examination showed that it might be due to the combined effects of membrane proteins and polar lipids [116]. In terms of commercial application, milk phospholipids are well-recognized ingredients for infant milk formula (IMF), which represent the world's fastest growing functional food in recent years [36].

2.5.2 Skin care

Skin parameter enhancement examination has been performed in either *in vivo* or *ex vivo*, yielding positive results except for a non-effectiveness report under the set conditions [117], as illustrated in Table 2-5. Some of these benefits appear to be related to phospholipids, altering the hydration of skin and therefore increasing elasticity and resilience.

2.5.3 Anti-inflammatory in gastrointestinal development

Milk phospholipids have proven to be able to modulate inflammatory reaction and to protect against gastrointestinal leakiness, as illustrated in Table 2-5. Animal models and cell models have shown that the polar lipids fraction from MFGM affects infant gastrointestinal development. Milk phospholipids diet decreased gut permeability [118], altered distal gut microbiota and reduced serum lipopolysaccharide (LPS) [119], inhibited infectivity of rotavirus [73], and regulated the neonatal gut microbiome and promote intestinal development [120].

2.5.4 Antioxidant activity

Milk phospholipids act as both antioxidants and a pro-oxidants and sometimes are used to alleviate food oxidation. Anti-oxidative activity of phospholipids might be due to such mechanisms as metal-chelation, alteration of the location of other antioxidants, and regeneration of other primary antioxidants. However, phospholipids can also act as primary antioxidants and pose significant antioxidant activity to biological membranes (*i.e.* meats),

owning to their unsaturated fatty acids and negative charge [121]. Phospholipid supplementation to soybean oil significantly retarded the oxidative process, extending oxidative stability index (OSI) from 7.62 to 12.96 h. However, phosphatidylcholine addition caused trimethylamine (TMA, fishy off-door) generation [122]. Marine lecithin (*i.e.* krill oil) consists of a natural antioxidant (astaxanthin) and phospholipids bound LC-PUFA, which inhibits oil peroxidation during its shelf life [123]. α-tocopherol enhanced the oxidative stability of marine phospholipid emulsions [124].

Table 2-5: Health effects of milk phospholipids.

Functionality	Dietary supplementary	Model	Result	Reference
Cognitive	PL PUFA	*In vivo*: Healthy elderly	Cognitive function was enhanced	[125]
Cognitive	Lacprodan® PL-20	*In vivo*: Healthy elderly	Set protocols to assess Lacprodan® PL-20	[126]
Cognitive	MFGM	*Ex vivo*: Rats	MFGM altered brain lipid	[127]
Cognitive	Lacprodan® PL-20	*Ex vivo*: Neonatal piglet	Spatial ability was enhanced	[115]
Cognitive	MFGM	*Ex vivo*: Suckling rat pups	Increased expression of brain gene BDNF and GLP-1R	[128]
Cognitive	Milk SM	*In vivo*: Low birth weight infants	SM activated prefrontal cortex of the brain, increasing score on visual evoked potential, attention, and memory	[129]
Cognitive	Ganglioside	*In vivo*: 6-months infants	Cognitive score increased	[130]
Cognitive	MFGM	*In vivo*: Infant and Toddler	The diet led to similar cognitive score to breastfed infants but showed higher score to pure polar lipids fed infants	[116]
Cognitive	Milk PL	*In vivo*: 54 healthy, non-obese adult men	Cognitive performance was improved under conditions of psychosocial stress but failed to moderate cortisol response	[113]
Cognitive	Milk PL coated dietary lipid	*Ex vivo*: Healthy male mice	T-maze test: Increased to 87% from 74% in short-term memory tests; while same in long-term memory	[114]
Skincare	Milk PL	*Ex vivo*: Dog with allergic skin disorders	Enteric improvement and enhancement of skin conditions	[131]
Skincare	Milk PL	*In vivo*: Healthy adults aged 20 to 39 year	Skin elasticity in the region below the eye increased	[30]
Skincare	Milk PL	*In vivo*: Atopic dermatitis patients	Not effective	[117]
Skincare	Milk PL	*Ex vivo*: Mice	Modulated epidermal covalently bound ceramides associated with formation of lamellar structures and alleviated skin inflammation	[132]
Anti-inflammatory	MFGM rich diet	*Ex vivo*: Mice	Attenuated the inflammatory response to a systemic LPS challenge; cut gut permeability.	[118]
Anti-inflammatory	Milk SM diet	*Ex vivo*: high-fat-fed mice	Altered distal gut microbiota and lowered serum LPS	[119]
Anti-inflammatory	Milk SM diet	*Ex vivo*: high-fat-diet-induced mice	Suppressed metabolic indicator of obesity	[133]
Anti-inflammatory	Milk PL extract	*In vitro*: MA-104 cells of embryonic African green monkey kidney	Polar lipids displayed effects of anti-rotavirus activity by focus-forming unit (FFU) assay	[73]
Anti-inflammatory	MFGM	*Ex vivo*: Rat pups	Protective and replenishing effects on neonatal intestinal epithelium caused by clostridium difficile toxin; milk PL deficiency led to defect of GI development	[120]

2.6 Structure, composition and occurrence of milk fats

2.6.1 Molecular structure, composition and occurrence

Bovine milk lipids are comprised of 97.5% TAG, 0.36% DAG, 0.027% MAG, 0.027% FFA, and 0.6% phospholipids [134]. There are also some minor lipid classes present in milk, for instance, sterols, carotenoids, lipophilic vitamins, and flavour compounds [18].

The triacylglycerol molecule consists of a glycerol backbone and three fatty acids esterified at the positions of *sn*-1, *sn*-2, and *sn*-3. Two subclasses of phospholipids are glycerophospholipids and sphingolipids. Glycerophospholipids consist of a glycerol moiety with two fatty acids esterified at the positions of *sn*-1 and *sn*-2 and a hydroxyl group at *sn*-3 position, linked to a phosphate group and a hydrophilic residue. The structural details of the hydrophilic residue determine the types of glycerophospholipids, namely phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidyl-glycerol (PG), and phosphatidic acid (PA) [19]. Sphingolipid consists of sphingosine backbone (ceramide, 2-amino-4-octadecene-1,3-diol), linked to a fatty acid through an amide bond and a polar head. Sphingomyelin (SM) is the predominant subclass of sphingolipids, having a phosphocholine head group. A minor constituent of sphingolipids in milk is glycosphingolipid, of which the polar group is comprised of carbohydrate groups (glucose, galactcose, and lactose) [13].

In intact raw bovine milk, lipids (3.3 – 4.6% [18]) are present in the form of milk fat globules (MFG), with an average diameter of 0.1 – 20 µm and are enveloped by a tri-layered phospholipid membrane [39]. The triple-layer membrane consists of a surface-active inner monolayer enveloping TAG in the centre and an outer bilayer in contact with the aqueous phase of the milk. The milk fat globule membrane (MFGM) is composed of polar lipids, proteins, glycoproteins, enzymes and minor neutral lipids [40].

2.6.2 Fatty acid profile

The most abundant milk fatty acids are palmitic (26.3 – 30.4%), oleic (28.7 – 29.8%), stearic (10.1 – 14.6%), and myristic (8.7 – 7.9%) acids [134]. Anhydrous milk fats (AMF), known by the US Department of Agriculture (USDA) as 1003, consist of palmitic acid (27.7%), oleic acid (26.5%), stearic acid (12.8%), and myristic acid (10.6%) [18]. Due to a high content of stearic and palmitic fatty acids (melting points at 69.3 °C and 62.9 °C, respectively), milk fats are solid at ambient temperature. Conjugated linoleic acids (CLAs) are isomers of linoleic

acids (0.8 – 2.5%) with the predominant CLAs being *cis*-9 and *trans*-11 CLAs (73 – 94%) [134].

SFA and monounsaturated fatty acids account for 62.2% and 28.9% (w/w) of the total fatty acids (FA) in the anhydrous butter oil of USDA 1003, respectively, whereas long-chain FAs (LCFAs, C13 – C21) accounts for 83.9% of the total FA, compared with medium-chain FA (MCFAs, C6 – C12, 8.8%) and short-chain FA (SCFAs, C2 – C5, 3.4%) [18]. Unlike LCFAs, SCFAs and MCFAs are absorbed intact as non-esterified fatty acids into the portal bloodstream and metabolized rapidly in the liver [135]. Via gastrointestinal digestion, medium-chain TAG is decomposed into glycerol and MCFAs, which reduces total cholesterol in serum by boosting hepatic synthesis of bile acid [136]. The SFA degrees of main shortening lipids are shown in Table 2-6 [18]. Lipids of dairy products can be separated by the Folch extraction [62], the Bligh method [63], the Röse-Gottlieb extraction [51], or dichloromethane method [61]. Total lipid (TL) content of samples may be measured using gravimetric determination, a Gerber-van Gulik butyrometer, infrared spectrometry in a Milkoscan FT2 apparatus [51], or gas chromatography [65].

Table 2-6: Composition of shortening lipids.

Code	Shortenings	TL (g)	SFA (g)	MUFA (g)	PUFA (g)	TFA (g)	SFA: UFA
4582	Canola oil	100.00	7.37	63.28	28.14	0.40	0.08
4506	Sunflower oil	100.00	10.30	19.50	65.70	-	0.12
4669	Soybean oil	100.00	15.25	22.73	57.33	0.68	0.19
4585	Margarine	80.32	14.20	30.29	24.17	14.95	0.26
4037	Rice bran oil	100.00	19.70	39.30	35.00	-	0.27
4615	Composite shortening	99.97	24.98	41.19	28.10	13.16	0.36
4002	Lard	100.00	39.20	45.10	11.20	-	0.70
1056	Cultured sour cream	19.35	10.14	4.59	0.80	0.80	1.88
1145	Butter	81.11	50.49	23.43	3.01	-	1.91
1003	Anhydrous butter oil	99.48	61.92	28.73	3.69	-	1.91
1017	Cheese cream	34.44	20.21	8.91	1.48	1.17	1.95
4513	Palm kernel oil	100.00	81.50	11.40	1.60	-	6.27
4663	Hydrogenated palm kernel oil (filling fat)	100.00	88.21	5.71	-	4.66	15.46
4701	Fully-hydrogenated soy oil	100.00	93.97	1.34	0.38	1.15	54.50

Notes: Saturated fatty acids (SFA), mono-, poly-unsaturated fatty acids (MUFA/PUFA), and trans-fatty acids (TFA) of shortening lipids per 100 g adapted from US Department of Agriculture (USDA) Database v.3.9.5.3 [18]; total lipids (TL)

2.6.3 Melting properties and solid fat index

Solid fat index (SFI) is a critical physical parameter of bakery shortening, determining the plastic behaviour of bakery fats and dependent on temperature. The SFI profile of milk fat crystal powder can be measured by pulsed nuclear magnetic resonance (p-NMR) with thermostatic incubation, and differential scanning calorimetry (DSC) can be used to determine the fat melting point [137]. The SFIs of major lipids in bakery products are illustrated in Figure 2-2: Solid fat index profile of typical shortenings for baked goods. [138]. The SFI profile of milk butter is very similar to that of general use margarine, all-purpose shortening, and cake lipids, and thus, milk butter is interchangeable with other shortenings. Cocoa butter can be used for coating bakery products, whereas milk fats are too soft for coating. Even as a cookie filler, milk fats are not firm enough and need to be formulated with other lipids. To achieve optimum bakery activity, bakery lipids should have 20% SFI at 25 °C and a minimum of 5% SFI at 40 °C [139]. For instance, a blend of stearin fraction of palm-based DAG and palm mid-fraction (50:50 w/w; SFI at 30% and 10% for 25°C and 40°C, respectively; polymorphic form β' + β; slip melting point 55.4 ± 0.12°C) makes a more effective bakery shortening than sunflower oil and palm oil [139]. An SFI profile of less than 15 – 20% at the dough temperature is too soft to make a shortening. However, fats that are too hard produce adverse effects, for instance, shortening with an SFI of *ca.* 47.5% at 20 °C produces less acceptable biscuits than shortening with an SFI of *ca.* 22.5% at 20 °C [140].

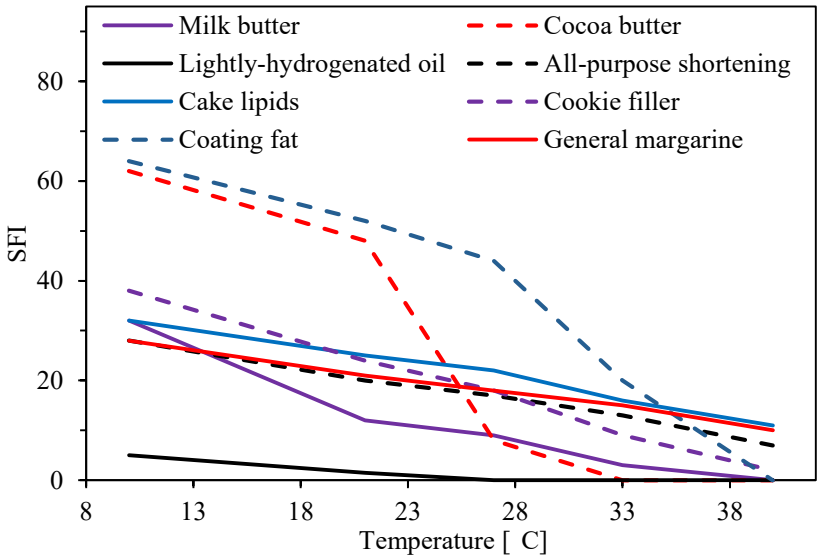


Figure 2-2: Solid fat index profile of typical shortenings for baked goods.

Notes: The Solid fat index (SFI) data was adapted from [138].

2.6.4 Crystalline polymorphism

In addition to SFI, polymorphic forms of milk fat crystals are also a factor in controlling the bakery activity of lipids [137] Lipids exist in three major polymorphic forms, α, β', and β, and their thermodynamic stability increases in the order of α < β' < β. Lipid crystal α is usually undesirable due to its instability. The β crystal (large plate-like) is stable, but coarse and sandy, whereas β' form is desired in baked goods since it is fine, needle-shaped, and stable. Enzymatic inter-esterification can rearrange fatty acids on the TAG backbone, creating tightly packed, small β' crystals, which produce more desirable bakery activity than composite blends [141]. Milk fats, together with other natural edible lipids such as tallow, palm oil, cottonseed oil, and high erucic acid rapeseed oil possess the β' polymorph, whereas crystals of soybean oil, sunflower oil, coconut oil, palm kernel oil, and lard are usually present in the β polymorph. Crystalline forms may transform into a more stable form as time and temperature change [142]. The crystalline polymorphism can be determined by using an x-ray diffractometer (XRD) [143]. Characteristic peaks of the acylglycerol emulsifier-shortening blend at 4.15 Å and 4.6 Å are from α and β forms, respectively, whereas the β' form demonstrates three signals at 3.8 Å, 4.2 Å and 4.3 Å. In addition, sub-β and sub-β' forms may cause peaks at 4.5 Å and 4.0 Å, respectively [144].

2.7 Milk fats in baked goods

2.7.1 Milk fats and related dairy products

Milk fats and related dairy products include butter, anhydrous milk fat, ghee, and cheese (combination of milk lipids and proteins) [145]. Butter is the predominant milk fat product used in the bakery industry, comprised of 81.11% milk lipids and 16.17% moisture, approximately (USDA 1145 in Table 2 [18]). In native milk, the enveloped fat globules are dispersed in the serum [54]. During churning, the membrane is disrupted and those milk fat globules aggregate to form butter, separating out from serum (buttermilk) [146]. Cultured lactic butter is more popular in Europe than in the USA, whereas sweet cream butter is more prevalent in the UK and USA than in other countries [147]. Salted butter (1.6 – 1.7% salt) has a 4-fold shelf life in refrigeration than unsalted butter due to reduced water activity [145]. Ghee is clarified milk fats from butter or cream, with an enriched flavour [148]. A milk butter blend with vegetable oil (*e.g.* corn oil, canola oil) reduces the overall SFA. Being hydrogenated from vegetable oils (*e.g.* soybean and palm oil) or animal fats (*e.g.* beef tallow) to raise the SFI and melting point, margarine is a cheaper substitute for milk butter in the bakery industry [149]. To avoid TFAs resulting from hydrogenation, inter-esterification of

vegetable oils (soybean oil, palm stearin, coconut stearin; 20:50:30, w/w/w) by Lipozyme RM IM, an immobilized *Rhizomucor miehei* lipase, produce SFI and crystals of β' polymorphic form, which are equally as effective as commercial margarines [150].

AMF contains 99.48% lipids and 0.24% moisture, respectively (Table 2-2 [18]). AMF and butter oil are comprised of no less than 99.8% and 99.6% lipids, respectively, without additives [145]. The AMF is produced by vacuum drying and removal of non-fat solids from pasteurized cream. First, cream (40% lipids) is concentrated to 70 – 80% milk fats, and after phase inversion, the milk fats are further dried to no more than 0.1% moisture [145]. The AMF can be produced from both butter and cream [54], and butter oil is made out of butter [145]. For cost-saving, substitution of 30% AMF by hydrogenated vegetable oils (*e.g.* soya or coconut oil) has been formulated into the shortening of bakery products in Asian countries such as Japan [138]. AMF has a broad melting and crystallization range, fully crystalizing at −40 °C and completely melting at 38 – 40 °C. Thus AMF can be fractionated into low (<10 °C), middle (10 – 20 °C), high (>20 °C), and very high (>50 °C for confectionery) melting fractions [151].

Cheese is produced from milk by inoculation with bacteria and separation of the resulting semi-solid curd (33 – 55% lipids for origin cream cheese, 0.5 – 16.5% lipids for reduced-fat cheese) from the liquid whey, leading to less-perishable products than milk [152]. Among the most commonly used bakery flavours, cheddar and parmesan cheeses have been used to impart flavour in biscuits or crackers [153]. In addition to flavour enrichment, cheese can also be used as a bakery coating or filling lipids [154]. A typical cheddar cheese contains 33.31% lipids (Table 2-2 [18]). Gas chromatography analysis of enzymatically-modified white cheese for bakery flavour revealed 58 volatile compounds of seven chemical classes including alcohols (12), aldehydes (8), ketones (10), esters (8), acids (11) and hydrocarbons (9), among which most compounds were produced by metabolism of carbohydrates, milk fats and amino acids [154]. Kefir cheese culture fermentation yielded volatile compounds, for instance, diacetyl (*i.e.* major buttery aroma), acetaldehyde, ethanol, and acetone by probiotic bacteria like *Lactobacillus* spp. and yeasts (*e.g. Saccharomyces* spp. and *Kluyveromyces* spp.) [155]. Bacterial metabolism produces diacetyl, for instance, by *Lactococcus lactis* subsp. [156].

Sour cream, a critical bakery flavour improver, is produced by the moderate-temperature fermentation of cream, and it can also be made by the treatment of acid-producing bacterial cultures on pasteurized cream. Compared to cream, sour cream (typical lipid content 19.35% in Table 2-7 [18]) is thicker and more acidic, with a longer shelf-life [157].

Furthermore, milk fats are often consumed together with biscuits and breads (*e.g.* as fillers) [158]. Milk fat products such as butter and AMF are sometimes manufactured as flaked or powdered forms by spray chilling or spray drying, which are easy to disperse [142]. The typical composition of milk fats and related dairy products are illustrated in Table 2-7 [18].

Table 2-7: Proximate nutritional information of main milk lipid products.

Code	Milk Fats	Water (g)	Energy (kJ)	Protein (g)	Lipids (g)	Ash (g)	Carbo-hydrate (g)
1017	Cheese	52.62	1,466	6.15	34.44	1.27	5.52
1005	Cheese	41.11	1,553	23.24	29.68	1.85	2.79
1009	Cheddar cheese	36.37	1,684	22.87	33.31	3.71	3.37
1053	Cream	57.71	1,424	2.84	36.08	0.53	2.84
1056	Cultured sour cream	73.07	830	2.44	19.35	0.51	4.63
1145	Butter	16.17	2,999	0.85	81.11	0.09	0.06
1003	Anhydrous butter oil	0.24	3,665	0.28	99.48	-	-

Notes: The nutritional data was adapted from US Department of Agriculture (USDA) Database v.3.9.5.3 [18].

2.7.2 Functionalities of milk fats in baked goods: baking activity

Milk fats have been used to perform multifunctional roles in bakery products, for instance, as mouthfeel and flavour improvers, texture improvers, dough conditioners, and anti-staling agents [142]. In addition, milk fats can fulfil a wide variety of functions such as laminating and filling fats, coating or topping lipids, spray oil, and imparting flavour [147]. The functions of milk fats are dependent on the dose and the type of baked products. For instance, they play more strengthening roles in yeast-leavened bread dough than in cookie/biscuit dough or cake batter, whereas cake fats are highly recommended for aeration and whipping in batter agitation [159], and biscuit or cookie laminating fats are mainly responsible for crisping and puffy effects by textural improvement [159]. Almost half of the lipids in coconut oil (USDA 4047) are comprised of lauric acid (41.84%, T_m = 43.2 °C), but bovine milk fats (USDA 1003) contain only 2.79% lauric acid in comparison [18]. Therefore, milk butter is soft for bakery coating, and its SFI need to be changed by blending with composite.

Bakery shortening is defined as the ability of a fat to lubricate, weaken, or shorten the structure of bakery products, thereby providing tenderization effects and other desirable textural properties to bakery products [160]. During the mixing process of dough or batter, lipids interact with gluten and starch particles to strengthen their network, thus improve the gas retention of dough. Hence, bakery products become softened, resulting in consistent grain,

lubricated mouthfeel, enhanced heat transfer, and extended shelf life [23]. Shortening lipids are made from milk butter, animal fats (*e.g.* tallow, lard), or hydrogenated plant oils (*e.g.* palm oil) [159]. In contrast to standard shortening, lipids such as hydrogenated vegetable fats may be used to replace milk fats for bakery products, such as biscuits [161].

Laminated dough shortening has an SFI of 10 – 40% for the temperature range of 33 °C to 10 °C, causing a puffy texture for croissants, danishes, and pastries. Milk butter is a benchmark laminated dough preparation agent for appropriate SFI profile and β'-form crystal. Cheap alternatives include hydrogenated shortenings and inter-esterified fats, which lead to a trans-fatty acids (TFA) issue or less acceptable sensory quality [137].

Bakery lipids have their characteristic SFI profile, plasticity (processability), and antioxidant stability [162]. For instance, a coconut oil cookie filler is designed as a 59% SFI at 10˚C, 29% at 21.1˚C, and 0% at 26.7˚C onwards, with a melting point of 24.5˚C. In contrast, croissant shortening melts at 39 °C, with an SFI profile of 39% at 10 °C, 27% at 21.1 °C, 22% at 26.7 °C, 19% at 33.3 °C, and 18% at 43.3 °C [159]. Milk fat flavours have been attributed to volatile molecules, including branched-chain fatty acids, lactones, methyl ketones, aldehydes, and other minor compounds, which are originated from milk fats or produced during fermentation, lipolysis, or processing. Milk fat products, such as cheese (*e.g.* cheddar and feta), cream, sour cream, and butter are all used to improve the sensory properties of bakery products [155].

2.7.3 Interactions of milk Fats with other bakery ingredients

Lipid-protein and lipid-starch interactions

Lipid-protein binding interactions can increase gluten polymerization. However, the ionic amphiphilic binding will cause interface aggregation due to charge neutralization, and therefore, these interactions may also decrease surface activity as the lipid concentration at the aqueous – oil interface increases to a certain level, which leads to the disruption of protein – protein interactions in the interfacial film [163]. The gluten – lipid interactions yield a dynamic balance of surface activity, altering the surface activity and aeration ability. This mechanism is critical for dough rheological characteristics and product textural properties. Horra *et al*. [164] compared refined milk fats (SFI 38% at 25 °C) and margarine shortening (SFI 5 – 25% at 25 °C) and found, through confocal microscopy, that the gluten network with milk fats is less developed and more orderly structured (with isolated starch particles) than the

network formed with margarine shortening, thereby producing greater elastic and viscous moduli, and higher puff pastry.

During dough mixing, milk fats coat the gluten network and starch particles, reducing the water hydration capacity of the dough [20]. With the formation of an extensible gluten film by hybrid hydration and lipid coating, the lipid crystals decrease the surface tension of the gluten film (lubrication effects), promoting aeration of the dough [23]. The crystals align their orientation along the air cells and stabilize them. Milk fats (β' polymorph) aerate more effectively than soybean oil (β polymorph) by forming fine and consistent gas bubbles [165].

During dough fermentation and proofing, the fat crystals further melt and become absorbed at the gas-liquid interface with increased temperatures. They re-orientate along the interface plane and hold the yeast-leavened carbon dioxide in the gas cells [166]. Low melting fats or oils have been found to be much less effective in gas retention at this stage [167].

During baking, starch particles become gelatinized and the gluten film turns into a permanent cross-linked thin film together with lipids, with the crust drying and browning (the Maillard reaction) taking place concomitantly [168]. Without lipids, the bubbles tend to coalesce or collapse and produce coarse crumb grain, whereas shortening fats lead to fine crumb grain and consistent porosity [169]. Hydrogenated fats produce a stronger dough and more tender cookies than sunflower oil [170].

When baked products cool down, amylose and amylopectin crystallize and retrograde in the early stages of storage and over the course of shelf life, respectively [168]. Using low-frequency NMR, it has been found that water migrates from crumb to crust or to amylopectin during storage, and immobilization of moisture will reduce water activity and decrease crystallization of amylopectin, thereby inhibiting the staling rate [171]. Most bread staling mechanisms have been explained by water migration. Reducing its water activity can slow down the staling rate of bread crumb. During bread storage, starch polymer retrogrades concomitantly with fat re-crystallization [172]. This concurrent polymorphic conversion from β' to β has been evidenced by powder XRD analysis for croissant samples [173].

In brief, shortenings such as milk fats have effects on the lubrication/stabilization (mixing), gas retention (proofing), textural tenderization (baking), and anti-staling (storage) properties of bakery products. The addition of emulsifiers can consolidate the above effects, thus reducing the amount of shortening lipids required. The interactions of lipids with other

ingredients is different among breads, cookies, biscuits and cakes, such as with the additional interactions of lipids with egg components.

Starch-lipid complexes

Starch can form complexes effectively with MAG, as it can with fatty acids, but TAG does not form complexes with starch [174]. A previous report has shown that four kinds of lipids: monopalmitate glycerol (96.3%), (palmitic acid (41.8%), dipalmitate glycerol (DPG, 1.1%), and tripalmitate glycerol (8.3%) have reduced complexing ability [174]. The starch-lipid complexes have been found to lower the glycaemic load of bakery products and impact on their staling processing. Using confocal laser scanning microscope (CLSM) and scanning electron microscopy (SEM), both non-inclusion and inclusion lotus seed starch-lipid complexes have been identified, causing slow digestibility of starch [175]. The complexing index of debranched starch-stearic acid complexes reached 89.31% [176], while that of the native starch (yam)-palmitic acid complexes (2%, w/w, starch base) was maximized as 26.39% [177]. In addition to the reduction of the starch glycaemic index, high amylose corn starch-lipid complexes inhibited the staling process of baked goods [178], as also evidenced by a recent report, where the firmness of wheat bread during storage was significantly reduced by resistant starch [179].

Emulsification lipids during dough or batter forming

Emulsifiers can be used to disperse milk fats, enhance their baking activity, assist ingredient mixing and emulsification during dough or batter formation, and promote aeration and air distribution, especially for cake batter [142]. Commonly used emulsifiers for baked goods include MAG and DAG (E471), lecithin (E322), sodium stearoyl lactylate (SSL, E481), and diacetyl tartaric acid ester of mono- and diacylglycerols (DATEM, E472e) [25]. For instance, in a high-ratio layer cake, 5% MAG was formulated into the shortening [144].

Similar to the baking activity of shortening, anionic emulsifiers such as DATEM, SSL, and calcium stearoyl-2-lactylate (CSL) are useful in both dough strengthening and bread softening, as are the nonionic emulsifiers (sucrose esters of fatty acids (SE), polysorbate-60 (poly-60)). Lecithin and distilled MAG have no strengthening effects [26]. Fu *et al*. [144] compared distilled MAG and four acylglycerols (40%) of octanoic acid (8:0), palmitic acid (16:0), stearic acid (18:0), and linoleic acid (18:2) and found that monopalmitate glycerol and monostearin glycerol led to a higher SFI and finer crystals (β' form), thus increasing aeration ability in batter formation and tenderizing the crumb of layer cakes. In contrast, monooctanoic glycerol and linoleic acid glycerol produced adverse effects to the SFI and β' form crystals,

thereby reducing cake size and increasing its firmness. In addition, lecithin and distilled monostearate stabilized the shortening crystals and increased the air-absorbing ability on both beef tallow and hydrogenated palm oil [180]. Using digital imaging of crumb micro-structure, the emulsifier functionality in assisting air aeration was recognized. At the same level of dough hydration, five emulsifiers (DATEM, SSL, distilled MAG, lecithin, and polyglycerol esters of fatty acids (PGEF)) increased the bread dough permeability and gas retention ability, resulting in increased gas bubble number and homogeneity [181].

2.8 Milk fats in baked goods

Breads use less fats and sugar than biscuits and cakes, and biscuit recipes use less water than breads and cakes. For instance, breads (AACC 10.10 recipe, flour based) are comprised of wheat flour, 6% sugar, 5% milk butter, 1.5% salt, 1.5% yeast, and 60% water; biscuits (AACC 10.54) consist of wheat flour; 42% sugar, 40% shortening, 1% skim milk powder, 1.25% salt, 1% sodium bicarbonate, 0.5% ammonium bicarbonate, 1.5% high fructose corn syrup, 22% water; and high-ratio cakes (AACC 10.90) are made out of wheat flour, 140% sugar, 50% shortening, 2% emulsifier, 12% dry skimmed milk powder, 5.5% baking powder, 9% egg white powder, 3% salt, and *ca.* 135% water [182].

2.8.1 Bread fats

Milk fats account for 3 – 4% of a bread recipe [182]. During dough mixing, both starch particles and gluten become hydrated, and the gluten proteins polymerize through reactions between the sulfhydryl (– SH) groups and disulphide (– SS –) bonds, forming an extensive, interlinked dough skeleton [183]. Milk fats mainly perform three kinds of functions in bread dough. First, lipid crystals brace the developed gluten network as a plasticizer. In this instance, shortening oil (*e.g.* sunflower oil) exhibits far less effect on the developing strong gluten network than shortening fats and milk butter due to less SFI [170]. Secondly, lipid crystals align themselves with the gas-liquid interface of bubbles during dough mixing, exerting lubricating effects [184]. Lastly, lipid crystals enhance the stability index of bread dough [185], and β' crystal-stabilized bubbles are larger than that of β crystals [186]. β' crystal lipids aerate dough more effectively than β crystal lipids [187].

During dough fermentation at 40˚C, yeasts produce carbon dioxide and ethanol. Newly produced carbon dioxide diffuses into gas bubbles and leavens dough to 1–1.5-fold in height [188]. Milk fats will then melt and form an extensible thin film, further stabilizing the gas bubbles [189], whereas doughs with insufficient lipids will leak gas via the gluten network

due to the penetration or rupturing of the cell wall [189]. However, a high concentration of lipids will inhibit dough rising, as aggregated gluten and solid lipid crystals exert low elasticity, thereby hindering the expansion of bubbles [174].

Upon heating, the cells expand with carbon dioxide diffusion and moisture/ethanol evaporation. With the moisture mobilization and heat transfer, gluten and gelatinized starch become solidified and form a fine crumb texture, and at the same time, the bread crust dries and turns brown due to the Maillard reaction [184]. Shortening fats melt fully and form an elastic thin film together with gluten along cell walls, again stabilizing gas cells [166]. Solid fat-incorporated breads exhibit increased porosity, loaf volume, and softness [190]. During baking, with the melting and gelatinization of starch particles, fat globules melt and form gas cells. The dough moisture migrates towards the edges of gas bubbles to evaporate. Eventually, the bread forms an interlinked porous texture [191].

During the staling process, the bread crust becomes leathery and the crumb turns rigid and unresilient, in parallel to the losses of aroma and eating quality [192]. The migration of moisture across the crumb and crust leads to increased bread rigidity. In addition, amylose and amylopectin retrograde successively over shelf life [193]. Milk fats have sufficient SFI at ambient temperature, and thus, they are able to act as plasticizers to increase storage stability, as well as change the thermoplastic properties.

2.8.2 Biscuit fats

Biscuits are among the most consumed bakery products worldwide, and they are formulated with flour, fat, sugar, milk, water, egg (optional), and salt into a viscous dough, and are baked on a flat surface [182]. In addition to lubrication and aeration in dough forming, biscuit fats perform roles including filling, laminating, coating, surface spray, nutritional value, sensory, and tenderization. Fats (T_m *ca.* 33 °C to give smooth mouthfeel, SFI 53% at 20˚C and 3% at 35 °C) constitute around half of the biscuit filler, in which inappropriate melting points will cause brittleness or filling collapse. Coating fats are usually cocoa butter equivalents (T_m *ca.* 36.6 °C), whereas typical spray lipids approximately possess SFI profiles of 22% at 20 °C and 0.5% at 35 °C [142]. Milk fats can be formulated compositely to fulfil these roles. Milk butter (no less than 7%, flour based) and cheese have been used to make premium butter biscuits (USDA 18214 [18]) and cheese crackers (USDA 45080543 [18]). Furthermore, cookies also utilize milk butter powder to laminate the dough sheets into several discrete layers, creating a puffy effect on the end product [142]. Enzymatically hydrolysed or cultured milk fats have

been used as flavour agents [154, 156]. In addition to the above functionalities, milk butter also serves as a nutritional ingredient. For instance, AMF and butter is comprised of polyunsaturated fats and lipophilic compounds such as vitamin E and β-carotene [147]. In addition, milk fats are natural lipids, without the trans-fatty acid issues such as hydrogenated shortenings [194].

Crackers are usually salty biscuits, based on layered dough, whereas cookies are normally made out of high fat and sugar recipes (short-dough [195], more cake-like). To counterbalance gluten development with syrup, comparable fats are added to confine starch granule swelling and limit dough forming [142]. Cookie dough is short-formed, and therefore a chemical leavening agent is used to increase its volume. The lipid content of leavened cookies and crackers is 7 – 20%, whereas unleavened cookies can have a lipid content as high as 16 – 33% (dough-based, Table 2-8). A typical cracker recipe incorporates 23.1% of milk butter (flour-based) [147]. In contrast to breads (35 – 45% moisture), the moisture content of cookies and biscuits are comparably low. For instance, crackers and cookies in Table 2-9 contain 2.75% and 5.9% moisture, respectively [140], and thus they can sustain a long shelf life. Compared with cookies, cracker recipes have no sugar (Table 2-8).

Table 2-8: Biscuit recipes based on 100 g flour.

Ingredients (g)	Cracker 1	Biscuit 1	Biscuit 2	Biscuit 3	Biscuit 4
Wheat flour	100.00	100.00	100.00	100.00	100.00
Water	27.50	35.71	13.33	20.00	20.00
Shortening	10.50	13.84	44.89	39.90	66.00
Baking Powder	0.80	0.98	1.11	0.50	-
Salt	1.00	0.66	0.93	0.71	2.40
Emulsifier	2.75	0.59	5.00	0.51	1.00
Sugar	-	26.79	60.00	40.40	33.00
Shortening Dough Base	7%	8%	20%	20%	33%
Reference	[196]	[197]	[198]	[195]	[199]

Table 2-9: Nutritional information and fatty acid profile of baked goods.

Baked Products	Bread	Biscuit	Cookie	Sponge Cake	Pound Cake	Wheat Cracker	White Cake	Yellow Cake
USDA code	18064	21142	3213	18133	45209528	18232	45262644	45174254
Water (g)	35.25	27.88	5.90	29.70	26.25	2.75	24.69	18.83
Energy (kJ)	1145	1547	1812	1213	1516	1903	1654	1725
Protein (g)	10.67	7.08	11.80	5.4	3.75	7.3	2.47	2.35
TL (g)	4.53	18.92	13.20	2.7	15.00	16.4	18.52	16.47
Ash (g)	2.01	3.31	2.00	1.2	-	2.83	-	-

Carbohydrate (g)	47.54	42.82	67.10	61	55.00	70.73	54.32	62.35
Sugar (g)	5.73	3.88	24.2	36.66	40.00	6.9	-	-
TDF (g)	4.00	2.50	0.20	0.5	-	15.48	43.21	49.41
SFA (g)	0.70	11.80	2.35	0.80	3.75	3.21	7.41	8.24
MUFA (g)	0.61	2.49	5.99	0.95	0.00	3.47	-	-
PUFA (g)	1.62	2.20	2.88	0.45	0.00	8.474	-	-
TFA (g)	0.03	0.21	0.02	-	3.75	0.034	-	-

Notes: data retrieved from US Department of Agriculture (USDA) [18]; total lipids (TL); total dietary fibre (TDF); saturated fatty acids (SFA); monounsaturated fatty acids (MUFA); polyunsaturated fatty acids (PUFA); trans fatty acids (TFA).

2.8.3 Milk fats in cakes

Cake batter is an emulsion of flour, sugar, shortening, egg, and other minor ingredients [200], and cakes contain more lipids and sugar than breads. In contrast, milk fats, especially butter, play a greater role in cakes than in biscuits. Yellow cakes (Table 2-10) use butter and whole egg, resulting in a rich colour, tender grain, and milky flavour. White cakes, on the other hand, usually use egg white and shortening instead of milk butter (Table 2-10). Pound cakes require equal amounts of flour, whole egg, milk butter, and sugar (Table 2-10), leavened by baking powder. Distinctly, butter may be absent in sponge cake recipes, where egg performs the aeration function in batters, creating foam and an airy grain.

In a layer cake formula ([201], Table 2-10), the amount of sugar is not greater than the quantity of wheat flour (both 100 g), and the egg amount (49.1 g) is equal or comparable to the amount of shortening (40.91 g). The amount of liquid in the recipe (114.89 g, Table 2-10) may be equal to or greater than the amount of sugar in the recipe (100 g, Table 2-10) [202]. Conversely, high-ratio cakes use more sugar than wheat flour (Table 2-10). To counterbalance the inhibitory effect of excess sugar on starch gelatinization [202], an extra egg is added to strengthen the formula (60 g egg *vs.* 40 g shortening [144] in Table 2-10).

Cake fats perform similar functions to bread lipids, for example, air incorporation, air cell stabilization, structure tenderization, and elevation of oven spring [203]. Propylene glycol monostearate (PGMS, 1.8% w/w), glycerol monostearate (GMS, 1% w/w), and lecithin (0.8% w/w) blended with soy bean oil are equally as effective as commercial liquid shortenings in increasing cake size and softness. However, liquid shortening cakes exhibit a reduced firming rate compared with cakes containing plastic shortening, seen over the course of three-weeks in storage [203].

Table 2-10: Cake batter recipes based on 100 g wheat flour.

Ingredients (g)	White Cake *	Yellow Layer Cake	High Ratio Cake	Pound Cake	Sponge Cake	Sponge Cake
Wheat Flour	100	100	100	100	100	100
Granulated Sugar	136	100	120	100	81.82	100
Water	106	65.79	75.00	16.33	-	-
Fresh Egg	60	49.10	60	100	127.27	100
Butter or Equivalent	25	40.91	40	83.55	86.36	100
Emulsifier	0.30	-	2	0.12	-	-
Skimmed Milk Powder	9	8.18	7	-	6.05	6.66
Vanilla Flavor	-	2.03	-	-	-	-
Salt	3	2.03	3	1.63	-	-
Baking Powder	6	0.55	5.5	6.53	9.09	-
Fats in Batter	6%	11%	10%	20%	19%	25%
References	[147]	[201]	[144]	[204]	[205]	[206]

Notes: * White cakes use egg white (not yolk) and shortening, instead of butter.

2.9 The replacers of bakery milk fats

To make cost effective bakery products, milk fats need to be replaced with more economic sources. In addition, milk fats are high-calorie (3665 kJ/100 g for AMF in Table 2-7), highly-saturated lipids (*ca.* 66% in Table 2-6). With a high content of shortenings in biscuits and cakes, the total lipid content in some bakery products (1812 kJ/100 g in Table 2-9) catalogues them as high-calorie foods (>1675 kJ/100 g [207]), as shown in Table 2-9. To produce bakery goods that are low in calories and saturated lipids, milk fats need to be substituted. There has been interest in using resistant starch (RS) emulsions to substitute bakery fats by 25 – 50%. In this regard, four forms of starch (2.6 – 46% RS) exhibited great potential in improving cookie/cake size and symmetry due to the extra hydration capacity of the added starches, while maintaining colour and sensory score [208]. Specialty fats (*e.g.* hydrogenated fats) in the bakery industry have been used to improve texture, shelf life and sensory acceptance. However, they are associated with high serum levels of low density lipoprotein and cholesterol, and the subsequent development of atherosclerosis [143]. Oleogels are recent alternatives to reduce SFA, as illustrated in

Table 2-11. It has been found that fat replacement has less impact on the acceptability of biscuits than sugar reduction [209].

Table 2-11: Fat replacer in bakery products.

Replacer	Baked goods	Replacement%	Result	Reference
Beeswax-sunflower oil oleogels	AACC 10.90 cake	100%	SFA 58%→15.5%	[210]
Candelilla wax-canola oil oleogels	AACC 10.54 cookie	30 – 40%	SFA 63.4%→32.3%	[162]
Carnauba wax-canola oil oleogels	AACC 10.90 cake	25%	SFA 74.2%→ 64.24%	[211]
Candelilla wax-canola oil oleogels	AACC 10.52 cookie	100%	SFA 52.8%→8.5%	[212]
Inulin from chicory roots.	Sponge cake: 100% sugar, 46% sunflower oil	70%	Reduced fat and fortified fiber	[213]
Inulin	Short dough biscuit: 74.1% margarine; 37% sugar	25%	Textural and sensory properties maintained	[214]
Inulin	Short dough biscuit: 30% shortening; 15% sugar	20%	Weakened lubrication of biscuit dough	[215]
Acetylated rice starch	Cookie: 60% sugar, 30% shortening	20%	Native and modified rice starch equally effective	[216]
Inulin	Biscuit: 45% margarine, 26.7% sugar	20%	Biscuit energy density reduced by 580 kJ/kg	[217]
Corn fiber, maltodextrin or lupine extract	Short dough biscuit: 132% margarine, 66% sugar	30%	28.6% fat reduction and 23 g/kg fiber fortification	[218]
Carnauba wax (5% cotton oil oleogels)	AACC 10.90 cake	50%	SFI similar to shortening fats	[219]
Chia seeds mucilage (80.16% carbohydrate, 10.63–10.76% protein)	AACC 10.90 cake and AACC 10.10 bread	50% and 75%	51.6 – 56.6% fat reduction and protein fortification	[184]
High-oleic sunflower oil and inulin/β-glucan/lecithin	Biscuit: 34% sugar, 46% shortening	100%	Lecithin (3%, sunflower based) achieved similar sensory quality	[220]
Chia mucilage gel	Pound cake: 75% sugar, 30% shortening	25%	Higher replacement led to adverse effect to color and texture	[221]
Puree of canned green peas	full-fat chocolate bar cookies: 324% sugar, 134% shortening	75%	By sensory assessment	[222]
High oleic sunflower oil + wheat bran (1.9:1)	Cookie: 52% sugar, 33% shortening, 40% egg	100%	SFA: 54.6→24.5%	[223]
77.3:34:12.4:1.3 olive oil: water: inulin: lecithin	Cake: milk fats 35%, sugar 33%, egg 40%	50%	SFA: <39% TL: <19%	[224]

Notes: Solid fat index (SFI); saturated fatty acids (SFA); total lipids (TL); base: 100 g flour.

In contrast to the moisture-retention and staling-retardation effects of carbohydrate-based replacers, protein-based replacers perform functions as texturizers. For instance, milk whey protein concentrate has been compositely used to substitute fats [225]. In addition, enzymes can also reduce shortening use, by targeting the endogenous flour lipids. Fugal lipase, *e.g.*

Lipopan F, has been successfully developed to hydrolyse flour lipids to replace milk fats [190, 226, 227]. In another report, amylase-hydrolysed starch was used to replace shortening, and achieved a comparable loaf size and consistency, but lower springiness and softness [228]. In general, reduced-fat bakery products have shown poorer performance in regards to mouthfeel, flavour, and texture properties than standard bakery products [229].

2.9.1 Carbohydrate-based milk fat mimetics

Carbohydrate-based fat mimetics are the most common milk fat replacers, including plant polysaccharides, dietary fibre, and starch [230]. These fat mimetics have been initially designed to generate sufficient baking activity, such as moisture retention, texturizing, and mouthfeel, whereas yielding only half to a quarter of the total calories of fats. However, in terms of flavour, palatability, crumb consistency, appearance, and customer acceptances, these replacers are less effective than milk fats. Recently, dietary fibre (*e.g.* inulin [231] and pectin [232]) and other resistant starches (*e.g.* Emjel) have been added to cookie and cake recipes [208], and they achieved similar textural properties to full-fat bakery products. Pectin (Yuja pomace) gel substitution (10%, w/w) led to the same level of volume and textural properties as shortening cake (AACC 10.90), with increased softness and whiteness [232]. Inulin (*e.g. Agave angustifolia* fructans) replacement (20%) led to similar sensory properties and enhanced prebiotic activity [233]. Light microscopy images showed that, with the replacement of shortening fats with β-glucans from an edible mushroom in the batter recipe, the population of gas bubbles became decreased, with broader size distribution, which indicated the loss of stabilization by forming an interfacial lipid film along bubbles during batter forming [234].

2.9.2 Lipid-based milk fat mimetics

Unsaturated lipids or low-calorie lipids have been used to replace milk fats. For instance, replacement of butter in breads by rapeseeds caused a 91% reduction of low-density-lipoprotein-cholesterol in plasma [235]. Margarine is a cheap alternative to milk fats. However, the high water content of margarine limits its use in biscuit manufacturing. Animal fats have been used to inter-esterify with plant oils (*e.g.* canola oil) to prepare bread shortenings [236], and cookies prepared with oils were firmer than full-fat cookies [237], whereas shortening (palm oil) and emulsifiers together have produced cakes with a similar firmness to cakes prepared with fats [203]. Inter-esterified beef tallow caused slower crystallization than tallow, and brought about an SFI increment of approximately 11% and 5% at 25 °C and 40 °C, respectively, thus increasing cake size and textural consistency. Inter-

esterification of the beef tallow-palm medium fraction produced similar plasticity and operability of shortening to beef tallow [238]. The addition of MAG and tripalmitin induced the formation of a polymorphic β-form, accelerating the processing of crystal formation and reducing the size of crystals [239].

2.9.3 Emulsion-based milk fat mimetics

Oleogels have been prepared to structure vegetable oils for bakery products, to reduce SFA and trans-fatty acids from the diet, as illustrated in

Table 2-11. Oleogels were prepared by thermal dispersion of sunflower oil into SSL (7–13%, w/w) at 75 °C [143]. Candelilla wax-canola oil oleogels reduced cookie SFA to *ca.* 8%, without damaging eating quality [212]. In another study, beeswax-sunflower oil oleogels reduced SFA in cakes to 14 – 17% from 58% in full-fat cakes [210]. In a previous report, monoacylglycerol organogels and sunflower oil-loaded hydrogels were used to replace shortening fats (palm oil), by 81% [240]. MAG-sunflower/palm oil (0.5%/7%, flour based) water gels have been formulated into bread recipes (4.7% MAG, 55.8% oil, and 39.5% water, w/w/w) [241]. Edible oleogels enhanced nutritional profiles and healthy benefits [242], and showed important features, such as thermo-reversibility and thixotropy [243]. SSL (7%) has been used as a gelling agent to structure sunflower oil oleogels, creating a crystal network similar to that of TAG [143].

Gels of hydroxypropyl methylcellulose (HPMC)/sunflower oil produced more acceptable biscuits than milk fat, vegetable shortening, sunflower oil/xanthan gum, olive oil/HPMC, and olive oil/xanthan gum [244]. A 15% replacement of HMPC/inulin made crisper biscuits than full-fat shortening [245]. Biscuit dough formulated with an HPMC emulsion showed similar rheological properties to dough made out of shortening fats [246].

2.9.4 Whole foods or combined ingredients to replace bakery lipids

Whole foods, such as avocado, chia, and banana, have been used to replace bakery lipids. For instance, chia (*Salvia hispanica L.* oil content 30 – 40%, protein content 15 – 25%) is comprised of rich polyunsaturated fatty acids, such as ω-3 fatty acids (linolenic acid, 54 – 67%) and ω-6 (linoleic acid, 12 – 21%). A chia mucilage gel (25%) has been shown to be a feasible alternative for pound cake shortening [221]. The use of oatrim (100%), bean puree (75%) or green pea puree (75%) as fat replacers in biscuits have proven to be equally

effective, and avocado puree can replace half of the shortening in both cakes and biscuits [21]. Okra gum from an edible green fruit (flowing plant of the mallow family) has been identified as a fat replacer for reduced-calorie bakery products, improving the nutritional quality of baked goods [247]. Avocado purée as a full replacement of shortening fats has brought about an increase in MUFA by 16.51%. Substitution by half demonstrated comparable acceptability, whereas further fortification with avocado purée caused undesirable flavour and aftertaste, according to the tested panelists in the study [248].

2.10 Conclusion

2.10.1 Milk phospholipid vesicle properties and health benefits

In this review, milk vesicle properties and health impacts were addressed. As an emerging material in nutraceutical and bio-pharmaceutical, milk phospholipids show advantages over lecithin of soy and egg yolk in encapsulation efficiency. Recently, various kinds of liposomes have been prepared for enhancing the solubility and bioavailability of encapsulates. Phytosomes, more stable carriers than liposomes, should provide a further area to study. For instance, milk phospholipids have been proven to support cognitive development owning to their balanced distribution in phosphatidylserine and sphingomyelin, which was almost absent in soy and egg yolk lecithin. Apart from brain function, milk phospholipids have a role in skin care, due to their more saturated fatty acids, which lead to milky-white colour and stability.

In summary, milk phospholipids have prospective applications in nutritional delivery, infant formula and cosmetic for their vesicle properties and biological functionalities. As potential alternatives to traditional polar lipids from egg yolk and soy, milk phospholipids need to be efficiently produced in large-scale. Ethanolic extraction remains the most used lipid extraction process in dairy industry. Defatting with supercritical carbon dioxide or acetone are frequently used to further refine phospholipids from lipids.

2.10.2 Bovine milk fats in baked goods and their replacers

This review verifies the relevance and significance of milk fats in bakery products. Their roles include altering structural, rheological, nutritional, and sensory characteristics. The milk fats can be used for dough strengthening in bread making, texture softeners in cakes, and sensory improvers in butter biscuits. In addition, they can be used as cookie fillers, laminating fats, topping and coating fats in bakery products. The interactions of milk fats with flour gluten and starch particles provide dough strengthening and texture improving effects to bakery products. Appropriate fat substitution with the design of new matrices such as oleogels and

inulin gels can improve the nutritional value of bakery products by reducing the saturated fatty acid content and energy density, and by increasing the nutrient quality, without adversely affecting the textural and sensory properties. In addition, lipase treatment of flour lipids or milk fats can generate emulsifiers including monoacylglycerols, which may enhance the shortening effect of milk fats and thereby reduce shortening use. Milk fatty acid-wheat starch complexes may also be facilitated so as to reduce glycaemic response and increase the shelf-life of baked goods.

In the end, milk fats have performed multi-functions in both technological importance and nutritional value, especially for high-end, valued-added baked goods. With partial replacement of milk fats in bakery products to balance their saturated lipids, both nutritional quality and customer acceptability can be further improved.

Chapter 3: Materials and methods

3.1 Materials for phytsomes

Milk phospholipid concentrate (20.81% and 51.3% (w/w) for lipids and proteins, respectively) was donated by Tatua Co-operative Dairy Company Ltd. Lipase (L3126, porcine pancreas, type II, EC232-619-9), phospholipid assay kit (MAK122), α-tocopherol (SKU258024, 95.5%), L-ascorbic acid (A92902, 99%), bovine bile extract, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were procured from Sigma-Aldrich. IEC-6 cells were seeded from Tianjin Key Laboratory of Food and Biotechnology, Tianjin, China. All other reagents including ethylenediamine tetraacetic acid (EDTA) were of analytical grade.

3.2 Phospholipid isolation and determination

3.2.1 Extraction of milk phospholipids

Milk phospholipids were extracted using a modified Folch method. Milk lipids (20 g) were first dissolved in 450 mL methanolic chloroform (1:2, v/v) for laboratory propose. After 20 min of agitation, 0.74% saline solution (160 mL) was added to precipitate casein protein. Following another 20 min of agitating, the mixture was centrifuged at 4000 *g* for 30 min at 4˚C. Subsequently, the aqueous supernatant was withdrawn with a 5 mL pipette, and the solid layer was removed with a scraper. Milk lipids in the chloroform layer (denser bottom layer) were dried under vacuum. The lipids were defatted three times with acetone. The acetone-insoluble precipitate was lyophilized to obtain purified phospholipids [249].

3.2.2 Proximate chemical compositional analysis

Total lipid content of milk samples was analysed gravimetrically, while the contents of proteins, triacylglycerols (TAG), and phospholipids of each fraction were determined by the Bradford assay, a TAG enzymatic colorimetric kit (glycerine phosphate oxidase peroxidase (GPO-PAP)), and the phospholipid assay kit MAK122 (Sigma-Aldrich China).

3.3 Preparation of liposomes and phytosomes

3.3.1 Preparation of liposomes

Liposomes were prepared using thermal method [250], with minor supplementation—sonication. The mixture of milk phospholipids and encapsulates (*i.e.* ascorbic acid and α-

tocopherol) was agitated in aqueous phase at 80 ℃ for 60 min. Subsequently, the liposomal suspension was further treated with sonication for 10 min to gain fine droplets (Sonics and Materials, Inc., 240 W, 20 kHz). The samples were analysed immediately following preparation.

3.3.2 Preparation of phytosomes

Phytosomes were prepared using ethanol evaporation method [92, 251]. Phospholipids and ascorbic acid (10 mg) were mixed at five molar ratios (0.33:1 – 3:1) and dissolved in 10 mL of ethanol. The mixture was then agitated with a magnetic stir bar at 300 rpm and 45 °C for 3 h. Finally, the formulated complexes were obtained by evaporating the solvent under vacuum. The precipitated phytosomes were stored at 4 °C before use. Vitamin E phytosomes were made using the same procedure.

3.3.3 Complexing index of phytosomes

The complexing index (CI) of associated vitamins C/E (ascorbic acid or α-tocopherol) in phytosomes was indirectly determined from the equation of $CI = 1 - V_{uncomplex}/V_{total}$, where $V_{uncomplex}$ is the amount of uncomplexed ascorbic acid or α-tocopherol, and V_{total} is the total quantity of the antioxidants. The uncomplexed antioxidant was precipitated using a α-tocopherol and ascorbic acid-insoluble solvent (chloroform, available at laboratory) to dissolve the phytosomes and phospholipids. Subsequently, the remaining antioxidant precipitate was separated by centrifugation at 8,000 *g* for 45 min at 4˚C. The contents of ascorbic acid and α-tocopherol were quantified with the SolarBio Life Sciences assay kits BC1235 and BC1423, respectively.

3.3.4 Encapsulate efficiency of liposomes

The ascorbic acid added into the liposomes was measured indirectly. The liposomal dispersion (2 mL) was centrifuged at 8,000 *g* for 45 min at 4˚C. The unincorporated ascorbic acid in the supernatant was determined using the assay kit BC1235 (SolarBio Life Sciences). The encapsulation efficiency (EE) of ascorbic acid liposomes was calculated by Eq. 3-1:

$$EE = 1 - AA_{unencasulated}/AA_{total}, \quad (3\text{-}1)$$

where $AA_{unencapsulated}$ is the amount of non-encapsulated ascorbic acid, and AA_{total} is the total quantity of ascorbic acid.

3.4 Verification of phytosomal complexing

3.4.1 Fourier-transform infrared spectroscopy

The molecular interactions of phytosomes was investigated by Fourier-transform infrared (FTIR) spectroscopy. Phytosomes (1 mg pellet) and KBr powder (100 mg) were finely ground together in a mortar. The fine powder was then pressurized to 10 MPa with a hydraulic unit to form a thin layer crystal. A Varian 3100 FTIR spectrophotometer (Varian Inc., USA) was used to scan the spectrum of the crystal from 400 to 4000 cm^{-1} at a resolution of 2 cm^{-1} [252].

3.4.2 Ultraviolet spectrum analysis

Characteristic ultraviolet (UV) absorbance of phytosomes will change after phytosomal complexation. Therefore, UV absorption spectrometry has been used to verify the conformation of phytosomes [253, 254]. The UV spectrums of the phytosomes, their constituents, and the mixture in ethanolic dispersion were measured with a UV spectrophotometer (Thermofisher Evolution 201 model) over the wavelength of 190 – 400 nm at room temperature [254].

3.4.3 Differential scanning calorimetry analysis

The differential scanning calorimetry (DSC) thermograms of pallet samples (*e.g.* phytosomes lyophilisation powder) were obtained using a Q1000 DSC unit (TA Instruments, USA) [255]. The samples (3 – 5 mg) were first placed and sealed in aluminium crimp cells, and then each cell was heated from 30 to 300 °C at a rate of 10 °C min^{-1} under a constant nitrogen flow (60 mL min^{-1}). An empty pan was used as the reference. The acquired temperature-enthalpy data for samples was analysed using the TA Instruments Universal Analysis 2000 software (Version 5.5.24).

3.5 *In vitro* digestion

3.5.1 Simulated intestinal digestion

The milk phospholipid dispersion (10 mL, phytosomes or liposomes) was diluted with 30 mL of distilled water. The simulated intestinal fluid (SIF) was composed of KH_2PO_4 (0.68%, w/v; as specified in the US Pharmacopeial (USP) Convention), porcine lipase (1280 lipase unit per pot), and bile extract (200 mg per pot), and was adjusted to pH 7.0 using 0.1 M NaOH prior to its addition to each pot to initiate the hydrolysis reaction. Aliquots of the digesta were taken at 0, 20, 40, 60, 90 and 120 min for compositional analysis [7].

3.5.2 Analysis of ascorbic acid and free fatty acids

The ascorbic acid released during intestinal digestion was separated by centrifugation at 8,000 g for 30 min at 4˚C, and was quantified using the assay kit BC1235 (SolarBio Life Sciences). The lipase and lipids were incubated with constant stirring at 95 rpm for 2 h at 37 ºC. The milk lipids were extracted with ethanolic diethyl ether (1:1, v/v). Subsequently, FFA concentration was determined titrimetrically with 0.05 mol/L of ethanolic potassium hydroxide [256], using the equation of $FFA = (V_{KOH}) \times (M_{KOH}) \times (MW_{ML})/2$, where V_{KOH} and M_{KOH} were the volume (µL) and molarity of potassium hydroxide titrant (µM), respectively, and MW_{ML} was the molecular weight of milk lipids (triacylglycerols and phospholipids, 794 g/mol) [257].

The experimental data of lipids was fitted using a linear regression model ($-\ln(1 - x_{lipids})$ vs t, k as the slope of trend-line) with the initial concentration of fatty acids being zero. The reaction rate constant k was described by the equation: $kt = -\ln(1 - x_{lipids})$, where x_{lipids} was the conversion rate of the substrates (phytosomes and liposomes) to fatty acids [258].

3.6 *In vitro* absorption and cytotoxicity

3.6.1 Cell culture

The IEC-6 cells, from small intestinal epithelial cell line, were grown in Dulbecco's modified Eagle's medium (DMEM), which comprised of 10% (v/v) fetal bovine serum (FBS) and 0.5% (v/v) penicillin and streptomycin (both 100 U mL^{-1}). The cells were first incubated at 37˚C and 5% CO_2 until their confluence. The confluent cells were then washed twice with phosphate buffered saline (PBS) after a removal of culture medium. EDTA solution (1 mL, 1%, w/v) was used to detach the cells from the wall (1 min, 37˚C), followed by the addition of 1 mL of culture medium to neutralize the EDTA. The detached cells were then suspended with a pipette and transferred to a tube for centrifugation at 240 *g* for 3 min at 4˚C. The supernatant was discarded, and the cells at the bottom of tube were again dispersed with 1 mL of culture medium. The amount of the cells was determined with a cell counting plate. DMEM medium was replaced every three days.

3.6.2 The cellular uptake of ascorbic acid

The method of cellular uptake of ascorbic acid was adapted from a previous report [254]. The confluent IEC-6 cells (passage of 15 – 35) were seeded in culture dishes (50 mm in diameter)

at a density of 0.2 million cells per petri dish. After 24 h of treatment of phytosomal or liposomal dispersion (5 mL per petri dish, 1 mg/mL), ice-cold phosphate-buffered saline (PBS) was used to wash the IEC-6 cell monolayer twice, and then trypsin-EDTA solution (1 mL, 1%, w/v) was added to suspend the cells. Following incubation (1 min), the EDTA was neutralized by addition of 1 mL of the growth medium. The suspension was centrifuged at 240 *g* for 3 min to precipitate the IEC-6 cells. Pelleted cells were gently washed with ice-cold PBS and then re-suspended using a further 1 mL of ice-cold PBS. The cell suspension was sonicated at 20 kHz for 10 min at 4˚C (by ice-cooling). The solid cellular debris was separated from the aqueous solution by centrifugation at 8,000 *g* for 10 min at 4˚C, and the ascorbic acid content in the supernatant was quantified with the assay kit BC1235 (SolarBio Life Sciences).

3.6.3 Cytotoxicity

Phytosomal and liposomal toxicity was colorimetrically analysed using the MTT assay [91]. IEC-6 cells were seeded in 96-well, flat-bottom microplates at a density of 5,000 cells per well. The cells were incubated for 24 h (37˚C and 5% CO_2) to become confluent. The cells were then treated for 3 h in a dispersion of phytosomes or liposomes (2 µL per well, 0.025 – 0.2 mg/mL) supplemented with 20 µM MTT. Following incubation, the dispersion was gently removed with a 200 µL pipette, and the cells were carefully washed twice with cold PBS. Dimethyl sulfoxide (DMSO, 100 µL) was then added to each well to dissolve the formazan crystals inside the cells. The absorbance of each well at 490 nm was measured in a microplate reader. The relative cell viability (*CV*) was obtained by normalizing the absorbance of the treated cells (A_t) with that of the controls samples (A_c), using Eq. 3-2.

$$\mathrm{CV} = (\mathrm{A_c} - \mathrm{A_t})/\mathrm{A_c} \quad \textbf{(3-2)}$$

3.7 Materials of milk phospholipids

Milk phospholipid concentrate (20.81% and 51.3% (w/w) lipids and proteins, respectively) was donated by Tatua Co-Operative Dairy Company (Morrinsville, New Zealand). Lipase L3126, porcine pancreas type II (EC232-619-9, CAS 9001-62-1), bovine bile (B3883), and phospholipid assay kits (MAK122), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), dichlorodihydrofluorescein diacetate (DCFH-DA), thiobarbituric acid (TBA), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich China. A triacylglycerol assay kit was purchased from Nanjing Jiancheng Bioengineering Institute,

Nanjing China. Finally, HT-29 cells (human colorectal adenocarcinoma cells with epithelial morphology) were seeded in Tianjin Key Laboratory of Food and Biotechnology, Tianjin, China. The other chemicals used in this study were of analytical grade.

3.8 Extraction and purification of milk phospholipids

3.8.1 Milk phospholipids extraction

Milk phospholipids were extracted using the methods in section 3.2.1.

3.8.2 Solid phase extrraction

The milk phospholipid fractionate was further purified from neutral lipids by solid phase extraction (SPE) [78]. The lipid solution (100 mg phospholipids in 1 mL of n-hexane) was first loaded on a silica gel SPE cartridge (500 mg, 6 mL). After the column was washed with n-hexane, the non-polar lipids were eluted with 10 mL of n-hexane-diethyl ether (1:1, v/v). Next, the phospholipids were recovered by two steps of elution, firstly with methanol (10 mL) and followed by a solvent mixture of methanolic chloroform and water (5:3:2, v/v/v, 10 mL). The eluted phospholipids were finally dried under a gentle flow of nitrogen.

3.9 Chemical analysis

3.9.1 Proximate chemical compositional analysis

Total lipid content of milk phospholipids was gravimetrically analysed, and whereas the contents of proteins, triacylglycerols (TG), and phospholipids of each fraction were determined by the Bradford assay [249], the TG enzymatic colorimetric kit (glycerine phosphate oxidase peroxidase (GPO-PAP) [259]), and the phospholipid assay kit MAK122 (Sigma-Aldrich China) [70], respectively.

3.9.2 Thin layer chromatography

Milk phospholipids in chloroform solution (2 µL, 100 mg/mL) were pipetted to the bottom edge of a silica gel plate for thin layer chromatography (TLC). The subclasses of milk phospholipids were separated with a solvent mixture (chloroform, methanol, and water, 65:25:4, v/v/v). Each band of polar lipids was oxidized in iodine vapour to visualize as coloured spot [260]. Standard phosphatidylcholine (PC) by Sigma-Aldrich was used as reference phospholipid subclasses.

3.10 *In vitro* digestion

3.10.1 *In vitro* digestion by pancreatic lipase and fungal lipase

Milk lipid dispersion (10 mL, phospholipids or triacylglycerol) was diluted with 30 mL of distilled water. The simulated intestinal fluid (SIF) was composed of dipotassium phosphate (0.68%, w/v), 150 mM sodium chloride and 30 mM calcium chloride [7], and the fluid was adjusted to pH 7.0 using 0.1 M NaOH. Lipase (0.4%, w/v) and bile extract (0.5%, w/v) were added to each pot to initiate the hydrolysis reaction. Aliquots of the digestion were taken at 0, 20, 40, 60, 90 and 120 min for compositional analysis. The free fatty acids (FFA) were extracted with ethanolic diethyl ether, and then quantified titrimetrically with 0.1 M ethanolic potassium hydroxide [256].

3.10.2 Release kinetics of FFA from milk phospholipids

The experimental data were fitted using the model mentioned in the section of 3.5.2.

3.10.3 Choline cleavage from milk phospholipids

The phosphatidyl groups in milk phospholipids were cleaved by phospholipases D and A_2, and pancreatic lipases in 96-well, flat-bottom microplates. The free choline was quantified at 570 nm using the colorimetric assay kit MAK122 (Sigma-Aldrich China). The reaction rate constant was obtained using the same model used for fatty acid release, as described in 2.4.2.

3.11 Absorption and cytotoxicity

3.11.1 Cell culture

The HT-29 cells were grown as described in the section of 3.6.1.

3.11.2 Cellular uptake of phospholipids

The method of determining the cellular uptake of ascorbic acid was adapted from a previous report [91]. The confluent HT-29 cells (passage of 15 – 35) were seeded in culture dishes (50 mm in diameter) at a density of 0.2 million cells per petri dish. After 24 h of treatment of milk phospholipids dispersion (5 mL per petri dish, 1 mg/mL), ice-cold PBS was used to wash the HT-29 cell monolayer twice, and then trypsin-EDTA solution (1 mL, 1%, w/v) was added to suspend the cells. Following incubation (1 min), the EDTA was neutralized by addition of the growth medium (1 mL). The suspension was centrifuged at 240 *g* for 3 min to pellet the HT-29 cells. The supernatant was aliquoted to determine the milk phospholipid concentration (C_f), and the cellular uptake (CU) of milk phospholipids was then calculated by Eq. 3-3:

$$CU = (C_i - C_f)/C_i, \quad (3\text{-}3)$$

where C_i and C_f are the initial and final concentrations of milk phospholipids, respectively.

3.11.3 Anti-proliferation activity

Milk phospholipid toxicity was analysed colorimetrically using the MTT assay [91], as descripted in section 3.6.3. The relative cell viability (CV) was obtained by normalizing the absorbance of the treated cells (A_t) with that of the control samples (A_c), using Eq. 3-4:

$$CV = (A_c - A_t)/A_c. \quad (3\text{-}4)$$

3.12 Antioxidant activity

3.12.1 DPPH radical scavenging activity

The free radical, 2, 2-Diphenyl-1- picrylhydrazyl (DPPH) radical scavenging activity was measured as previously described [261]. Samples (0.25 mL of the various concentration) and freshly-prepared DPPH (1 mM methanolic solution, 0.18 mL) were added to methanol (2.57 mL), and were then incubated in dark for 30 min to measure the absorbance at 515 nm (DPPH) with a spectrophotometer. The DPPH radical scavenging rate (*SR*) was calculated against reagent blanks with Eq. 3-5:

$$SR(\%) = \left(1 - (A_t/A_c)\right) \times 100, \quad (3\text{-}5)$$

where A_t and A_c were the absorbance value of the samples and controls at 30 min, respectively [262]. Ascorbic acid was selected as the reference antioxidant.

3.12.2 ABTS radical scavenging activity

A colorimetric assay was used to quantify the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) radical scavenging activity of milk phospholipids [263], in comparison to ascorbic acid. The mixture (1:1, v/v) of ABTS solution (7 mM) and potassium persulfate ($K_2S_2O_8$, 2.45 mM) was kept for 16 h in the dark to form the radical cation ($ABTS^{\bullet+}$) at ambient temperature. Subsequently, the optical density of the solution was adjusted to 0.70 at 734 nm with ethanol. The mixture of the ABTS working solution (1 mL, 0.7 in absorbance) and samples (100 µL) was then incubated for 20 min and then the absorbance (A_{20}) was measured at 734 nm. The ABTS radical scavenging rate (*SR*) was calculated using Eq. 3-6, where A_{blank} is the absorbance of the reagent blanks:

$$SR(\%) = ((A_{blank} - A_{20})/A_{blank}) \times 100. \quad (3\text{-}6)$$

3.12.3 Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of the milk phospholipids was measured with a spectrophotometric protocol as described by Zhu *et al.* [264]. PBS (1mL, 0.4 M, pH 7.4), 1,10-phenanthroline (1 mL, 2.5 mM, Sigma-Aldrich), $FeSO_4$ (1 mL, 2.5 mM), and H_2O_2 (0.5 mL, 0.01%, w/v) were added to the milk phospholipid dispersion (1 mL of various concentration). After 1 h of incubation at 37˚C, the absorbance of the samples was measured at 536 nm (1,10-phenanthroline-Fe^{2+} complex). The scavenging rate (SR) of hydroxyl radicals was calculated as follows: $SR(\%) = [(A - A_b)/(A_c - A_b)] \times 100$, where Ac was the absorbance of the controls (the samples and H_2O_2 were replaced with 1.5 mL of distilled water), Ab was the absorbance of the reagent blanks (the samples were replaced with 1 mL of distilled water), and A was the absorbance of milk phospholipid samples.

3.12.4 TBARS formation inhibition method

Thiobarbituric acid reactive substance (TBARS) assay on milk phospholipids was determined by the description of Murdifin *et al.* [265], in comparison with both ascorbic acid (0.1 mg/mL) and butylated hydroxytoluene (BHT, 0.1 mg/mL). The mixture of 4 mL milk phospholipid dispersion (1 mg/mL) and 1 mL linoleic acid (13%, w/v) were dispersed with sonication for 10 min at 20˚C. Following encapsulation of the oil droplets, the samples were incubated in darkness at 50˚C for 2 days. An aliquot (1 mL) was added to 2.5 mL TBA solution (0.375% TBA (w/v), 15% trichloroacetic acid (w/v), and 0.25 M HCl), and was then boiled for 10 min. The thiobarbituric reactive substance, malondialdehyde (MDA), from the oxidation of linoleic acid, became pink. The supernatant after centrifugation (5,000 *g*, 25˚C, and 10 min) was then determined spectrophotometrically at 532 nm. Lipid peroxidation inhibition percentage (*IR*) was calculated by Eq. 3-7:

$$IR(\%) = [(A_c - A_s)/A_c] \times 100, \quad (3\text{-}7)$$

where A_c and A_s were the absorbance values of controls and samples, respectively.

3.12.5 Cellular antioxidant activity

The cellular antioxidant activity (CAA) of milk phospholipids was assayed on HT-29 cells [266]. The cells were seeded on a 96-well microplate, with a density of 5×10^4/well. After one day's incubation at 37˚C, the growth medium was removed and the cells were washed twice

using PBS. Subsequently, the cells were treated with milk phospholipid dispersion (0.05 – 1 mg/mL) and DCFH-DA (25 µM) for 60 min. Following the removal of growth medium and washing twice with PBS, 600 µM 2,2′-azobis (2-amidinopropane) dihydrochloride (ABAP, Sigma-Aldrich) was applied to the cells in 100 µL of growth medium. The emission at 538 nm was measured with excitation at 485 nm in one hour, using a microplate reader. The CAA value was calculated by Wolf *et al.* [266].

3.13 Dough preparation and bread making

3.13.1 Ingredients and reagents

Whole wheat flour, comprising 1.2% of lipids and 11.5% of gluten, was purchased from Champion Flour Milling Ltd, Christchurch, New Zealand. Bakery yeast was procured from Edmonds Yeast Company, Christchurch, New Zealand. Milk butter from bovine pasteurized milk consists of 82.9% and <1% for total lipids and proteins, respectively, and was supplied by Mainland Baking Company, Christchurch, New Zealand. Lipopan F (25 – 50 kLU g^{-1}, active at pH 5 – 7) was supplied by Novozyme Company, Australia. Pepsin (P1700 0.8 – 2.5 kU mL^{-1}) and pancreatin (P75458, USP specification) were procured from Sigma-Aldrich Australia. Amyloglucosidase was procured from Megazyme Australia, with enzymatic activity of 3.3 kU mL^{-1} on soluble starch.

3.13.2 Bread making

The bread samples including controls were prepared according to AACC 10.10 straight dough and yeasted formula [182], consisting of 150 g of whole wheat flour, 90 mL of water, 2.25 g of instant yeast, 2.25 g of salt, 9 g of sugar, and 7.5 g of milk butter. In lipase-treated milk lipid samples (LML4.5), the level of Lipopan F supplementation was 4.5 mg for 150 g flour. Whereas the doses for LML9 and LML18 were double and quadruple to that for LML4.5 samples, respectively. Whereas, for LFL2.3 samples, Lipopan F lipase concentration was as 2.3 mg per 150 g flour. With regard to lipase-treated milk lipid (LML) samples, microwave-melted butter was mixed with lipase in aqueous dispersion and incubated with constant rotation at 95 rpm for 20 min at 37˚C, and the lipolysate was added to other ingredients for dough mixing. Whereas, for lipase-treated flour lipid (LFL) samples, lipase in aqueous dispersion was added to wheat flour to digest its lipids during dough mixing and maturation at ambient temperature. After the dough was formed, it was removed from the mixer and kneaded to release the air trapped during dough mixing. The matured dough was either tested immediately or it was divided into 50 g portions and moulded in 110 mL baking pans. The

dough was allowed to rise by 1.5 – 2 times in volume during the 1 h fermentation period at 40 ºC. Finally, the fermented dough was transferred to a bakery oven for 15 min of baking at 180 ºC. The process flow of bread making and analysis was illustrated in Figure 3-1.

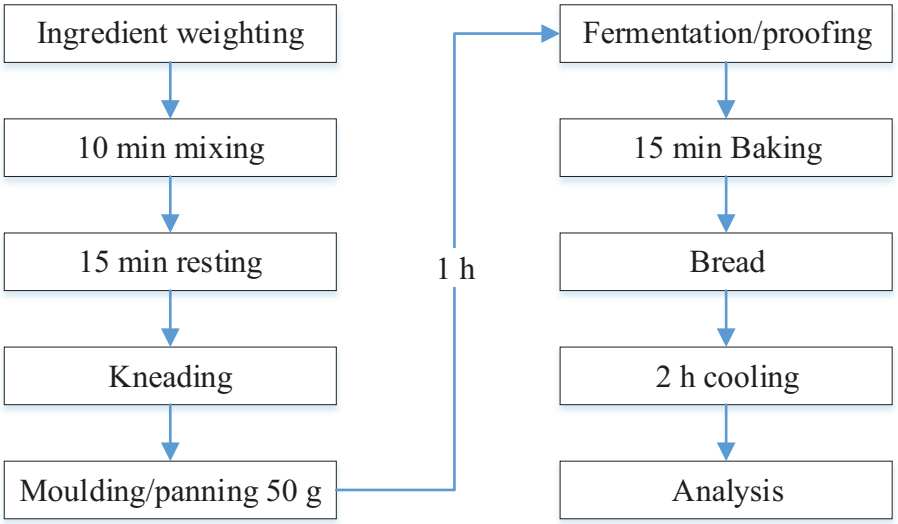


Figure 3-1: The process flow of bread making and analysis.

3.14 Dough characteristics

Dough characteristics were analysed by texture analyser (TA-XT2 TPA unit, Stable Micro Systems, Surrey, UK). All rigs and probes used for these analysis were supplied by Stable Micro systems, Surrey, UK.

3.14.1 Dough rheological properties

A Kieffer rig was used to measure rheological properties of the doughs, according to AACC method 54.30.02 [182]. After resting in a conditioned area for 20 min, the matured dough was rolled and placed in a Teflon mould, where it was extruded and stabilized for another 20 min before measurement. The configuration parameters were as described by Liu *et al.* [267].

3.14.2 Dough stickiness

The stickiness of dough, a factor affecting dough machinability, was performed using Chen-Hoseney stickiness rig. The matured dough was placed in a test cell for an extrusion to 1 mm in height, resting for 30 seconds to release extrusion stress before measurement. The adhesive force to separate a 25 mm diameter plastic probe from the extruded dough was recorded as the dough stickiness [267].

3.14.3 Dough firmness

Dough firmness was measured by penetrating with a cylinder probe of 6 mm in diameter into each dough pot. Prior to measurement, an aeration plunger followed by a flat plunger were applied to remove any air pockets and flatten the dough sample, respectively.

3.14.4 Dough pH

The dough pH was measured according to a method by the International Association for Cereal Chemists (ICC). Matured dough (5 g) was dissolved in 100 mL of 5% acetone aqueous solution (v/v) for 30 min before pH measurement [268].

3.15 Bread analysis

3.15.1 Physical properties

The loaf volume was determined on intact loaves according to AACC method 10.05.01 [182]. The specific loaf volume was obtained from the ratio of volume and weight. The porosity was calculated by the equation of $\varepsilon = 1 - \rho_{total}/\rho_{solid}$, where ε was crumb porosity; ρ_{total} represented the crumb density; and ρ_{solid} was the density (1.111 g/mL) of compact crumb without gas cells for wheat flour bread baked at 180˚C [269].

3.15.2 Crust colour

A CR210 colorimeter unit equipped with a 50 mm diameter was used to measure the colour parameters (CIELAB colour space: L*, a*, and b*). The whiteness index (WI, [270]) and brownness index (BI for evaluation of the Maillard reaction, [271]) were calculated with Eq. 3-8 and Eq. 3-9, respectively.

$$WI = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}}, \quad (3\text{-}8)$$

$$BI = 100(x - 0.31)/0.17, \text{ where } x = (a^* + 1.75L^*)/(5.645L^* + a^* - 3.01b^*). \quad (3\text{-}9)$$

3.15.3 Texture profile analysis

A texture profile analysis (TPA) unit (Texture Technologies Corp., Scarsdale, NY) was used to analyse bread texture profile according to AACC method 74.09 [182]. A 6 mm diameter cylindrical probe and a load cell of 5 kg, and TA.XT2 kit (Stable Micro System Ltd., UK) were equipped to the unit. The bread macro software provided by the TPA unit was used to collect the data. Bread samples were cut into slices of 2.5 cm thickness. The crust was

removed to restrict the limit to TPA analysis. TPA analysis was measured in triplicate from three different slices. The probe speed was set at 1 cm/s.

3.16 Bread *in vitro* digestion and glycaemic glucose equivalent

A dinitrosalicylic acid (DNS) colorimetric assay was used to determine the glycaemic glucose equivalent (GGE) of reducing sugar generated from starch *in vitro* hydrolysis [272]. Glucosidase enzymatic digestion was carried out in 70 mL plastic containers on a multi-magnetic stirrer. A GOPOD starch kit TSTA-100A (Megazyme Int. Ltd, Co. Wicklow, Ireland) was used to determine the total starch content of bread, according to AACC method 76.13.01 [182].

The potential glycaemic response of milk lipid-complexed starch gels was determined by simulated gastrointestinal digestion [272]. Starch gel (2.5 g) was added to 30 mL of distilled water to conduct *in vitro* digestion (37 °C with constant stirring). HCl (0.8 mL, 0.05 M) and 1 mL of pepsin (10%, w/w, Acros Organics, New Jersey, USA CAS: 901-75-6) was added to each digest to simulate gastric fluid. After 30 min of simulated gastric digestion, 1 M $NaHCO_3$ (2 mL) solution was added to neutralize the gastric acid, and 0.1 mL amyloglucosidase was added to prevent end product inhibition. This was followed by the addition of 5 mL pancreatin (2.5%, w/w, (EC: 232-468-9, CAS: 8049-47-6, activity: 42.36 FIP-kU/g, Applichem GmbH, Darmstadt, Germany) in 0.1 M sodium maleate buffer to simulate intestinal fluid and the digestion was continued for 2 hrs. During the intestinal digestion phase, aliquots of 1 mL were taken at 0, 20, 60, 120 min and added to 4 mL of ethanol for subsequent reducing sugar analysis by a dinitrosalicylic acid (DNS) colorimetric assay.

3.17 Complex index

Iodine solution was used to spectrophotometrically determine the CI value of starch-lipid by the equation of $CI = 1 - A_t/A_c$, where A_t and A_c were the absorbance (at 620 nm) of lipase-treated samples and controls, respectively [174].

3.18 Anti-staling performance

3.18.1 Water activity and bread staling during shelf life

The crumb water activity was measured with an AquaLAB CX2 unit (Decagon Devices, Inc., WA, USA), using the moisture diffusion method. The firmness of intact breads during storage was tested according to Giannone *et al.* [273].

3.19 Lipolysis and free fatty acid determination

The mixture of fungal lipase Lipopan F and milk butter dispersion was incubated with constant stirring at 95 rpm for 2 h at 37 ºC. The milk lipids were extracted with ethanolic diethyl ether (1:1, v/v). Subsequently, FFA concentration was determined as mentioned in the section of 3.5.2.

3.20 Materials and reagents for amylose-fatty acid complexes

Wheat starch (WS, S5127), corn starch (CS, S4126), waxy corn starch (WCS, S9679), rice starch (RS, S7260), and stearic acid were procured from Sigma-Aldrich, while high amylose corn starch (HACS) was obtained from Megazyme Australia. Milk lipids (bovine pasteurized milk butter, total lipids 82.9%) were supplied by Mainland Baking Company, Christchurch, New Zealand. Lipopan F fungal lipase was procured from Novozyme Australia. All other reagents were of analytical grade. The total content of proteins and lipids of milk butter were 52.51 ± 4.45 mg/kg and 0.86 ± 0.03 g/g, respectively. The amylose contents were 67.0 ± 0.2%, 29.74 ± 0.24%, 23.70 ± 0.96%, and 26.75 ± 0.33% for HACS, CS, WS, and RS, respectively. WCS was composed of pure amylopectin.

3.21 Preparation of starch-milk fatty acid complexes

Milk lipids (butter) were first hydrolysed by fungal lipase Lipopan F. The hydrolysates were lyophilized to make milk fatty acid dry pellets. Milk fatty acids at fortification concentration of 0 (control), 4, 12, and 20% (w/w, starch based) were mixed with 3 g of starch (WS, CS, RS, WCS, and HACS) in 25 mL of distilled water. The mixture was gelatinised using a RVA-4, 23-min standard-2 procedure, in which the mixture was heated from ambient to 95˚C at a rate of 5˚C/min and held at 95˚C for 7 min before cooling down to ambient temperature again. Stearic acid supplementation (12%, w/w, starch based) was used to compare with milk fatty acids.

3.22 Light microscopy analysis and particle size distribution

One gram of fine starch powder was dispersed in 30 mL distilled water and stained with 0.2% iodine solution (w/w) before observation and imaging with a Nikon H55S computerised microscope (bright field, phase contrast mode) [274]. The starch gels with entrapped milk fatty acids were dyed with Sudan black ethanolic solution (0.7 g in 100 mL ethanol) and analysed with the same microscope to starch particle analysis. PSDs of starch granules solutions were measured at approximately 10% laser obscuration using the laser diffraction

particle size analyser MasterSizer 3000 (Malvern Instruments Ltd., Malvern, Worcestershire, UK) equipped with a wet sample dispersion unit (Malvern Hydro MV, UK). The refractive indexes of starch and dispersant (water) were set 1.54 and 1.33, respectively. The surface-area-based mean diameter ($d_{3,2}$) and volume-based mean diameter ($d_{4,3}$) were also obtained together with PSD profile [275].

3.23 Gel rheological properties and CI measurement

The rheological properties of both untreated and fatty acid-complexed starch gels were determined with a Perten rapid viscosity analysis unit (RVA-4, FF Instrumentation Ltd., New Zealand), according to AACC 76.22 method [182]. The firmness of fresh gels was measured using a TA-XT2i texture analyser with a probe of 6 mm diameter, which penetrated 4 mm into the gel at a speed of 0.2 mm/s. The peak force at 4 mm compression cycle was defined as the gel firmness [276]. The CI value were spectrophotometrically determined as described in the section of 3.17.

3.24 *In vitro* digestion of starch-milk lipids and starch hydrolysis kinetics

The potential glycaemic response of milk lipid-complexed starch gels was determined by simulated gastrointestinal digestion [272]. The logarithmic slope (LOS) plotting of reaction curve was performed to determine lipolysis reaction rate constant [277]. The experimental data was fitted using a model described in the section of 3.5.2.

3.25 Spectroscopy analysis and thermography for starch-lipid complexes

A Varian 3100 FTIR spectrophotometer (Varian Inc., USA) was used to scan the spectrum of the milk fatty acid-complexed wheat starch crystal from 400 to 4000 cm^{-1} at a resolution of 2 cm^{-1} [252]. DSC was used to assess the thermal properties of starch-lipid complexes. Wheat starch-fatty acid mixtures (3 mg) were weighed accurately into aluminium pans of 40 µL, and 9 µL of distilled water was then added. After that, the pans were sealed and equilibrated for 30 min at room temperature. The enthalpy-temperature curve were recorded during the heating from 20 to 120 °C at a rate of 10 °C/min. An empty pan was used as the reference. The acquired data were analysed using the TA Instruments Universal Analysis 2000 Software [174].

3.26 Lipolysis and determination of free fatty acids

Free fatty acids (FFA) concentration was determined titrimetrically with 0.05 M of ethanolic potassium hydroxide [256]. FFA content (µg) was calculated as a previous method [257]. In

addition to FFA determination, the fatty acid profile of lipid was chromatographically analysed using fatty acid methyl esters (FAME) method, with a gas chromatograph unit equipped with a flame ionization detector (FID) [54].

3.27 Materials for milk lipid lipolysis

Corn starch (S4126), wheat starch (S5127), and rice starch (S7260) were procured from Sigma-Aldrich. Porcine pancreatic lipase (L3126, EC232-619-9, CAS 9001-62-1) was supplied by Sigma-Aldrich. Bovine bile (B3883) and amyloglucosidase were provided by Sigma-Aldrich USA. Milk butter (from pasteurised cream milk, proteins 0.6%, lipids 81.4%, carbohydrate 0.6%, sodium 0.6%, 30.3 MJ/kg) was procured from Pams, New Zealand. All chemicals used were of analytical grade. Total protein content of milk butter was 52.51 ± 4.45 mg per kg, as determined according to the Bradford assay [249], and whereas, milk butter was extracted using Folch method and the total lipid content was gravimetrically analysed [80]. After being dissolved in methanolic chloroform solution (10 mL, 1:2, v/v) with constant stirring at 120 rpm for 30 min, milk fat dispersion was then washed with saline solution (4 mL, 0.74%, w/v). Next, the mixture was centrifuged at 700 g for 10 min at 4˚C. Following the removal of supernatant, the chloroform layer at the bottom was then evaporated overnight with a gentle nitrogen stream. The dry precipitate was stored at –20˚C before its use as milk fat samples.

3.28 Proximate nutritional analysis

Total protein content of milk butter was determined by the Bradford assay [249], while total lipids of milk butter were analysed gravimetrically. The lipids (1 g) were dissolved in methanolic chloroform solution (10 mL, 1:2, v/v) with constant stirring for 30 min at 120 rpm. The lipid solution was then washed with saline solution (4 mL, 0.74%, w/v). Next, the mixture was centrifuged at 700 *g* for 10 min at 4˚C. Following the removal of supernatant, the chloroform layer at the bottom was then evaporated overnight with a gentle nitrogen stream. The dry precipitate was weighed to determine the amount of total lipids [278].

3.29 Preparation of milk lipid dispersion and its turbidity analysis

Sonication-assisted high shearing homogenisation was used to prepare the emulsion. Milk lipids (2.5 g milk butter) were dispersed in 30 mL of distilled water with constant stirring for 30 min at 50 ℃, followed by 5 min homogenisation at 16,500 rpm and subsequent 15 min ultra-sound treatment at 37 kHz [279]. The emulsion turbidity (T, m^{-1}) was calculated using equation of $T = 2.303 \times A \times D/L$, where A, D, and L were the absorbance at 500 nm,

dilution factor, and light path length, respectively [280, 281]. The emulsion stability index (*ESI*) of the milk lipid dispersion was calculated with the equation of $ESI = t \times A_0/(A_0 - A_t)$, where t is the time interval (*i.e.* 10 min), and A_0 and A_t were the absorbance at 500 nm of lipid emulsion at 0 and 10 min, respectively [282].

3.30 Preparation of starch gels to entrap milk fats and rheological analysis

Starch fat-filled starch gels were prepared with starch (2, 2.5 or 3 g; rice, corn or wheat starch, respectively), 1 g milk fat, and 25 mL distilled water [283]. The milk fat-loaded rice, corn or wheat starch gels (testing samples) were defined as RF-, CF- and WF-gel, respectively, and whereas, MFG (control samples) represented the naked milk fat emulsion. For side control samples, R-, C- and W-gels were denoted as rice, corn and wheat gel, respectively, without filling milk fat. Milk fat-contained mixture was homogenized using a high shear homogenizer at 16,500 rpm, followed with an AACC 76.21.01 Standard 2 heating program (23 min heating including 7 min holding at 95 °C) in the cartridge container of a rapid viscosity analysis unit with constant bladed-agitation at 160 rpm [284]. The milk fat-filled starch gels were instantly analysed for lipolysis reaction rate and degree. A crude oil-in-water (O/W) emulsion, as the control sample, was prepared from milk fat, water and 0.5% SDS emulsifier by 16,500 rpm homogenization at 55 °C, and the coarse emulsion was fine-tuned and stabilized with a 37 kHZ ultra-sonication unit for 10 min [279, 285].

3.31 Optical, confocal laser scanner microscopy

The starch gel light microscopy analysis and PSD analysis methods were as in the section of 3.22. Confocal laser scanner microscopy (CLSM) protocol was adapted from a recent report [215], and Sudan Black was used to stain milk fats at emission wavelength 488 nm and absorbance wavelength 515 nm. Fat droplet particle size distribution was analysed using both microscopy techniques [incl. light microscopy and confocal laser scanning microscopy (CLSM)] and laser diffraction technique. The microscopic images were processed to find out surface-area-based diameter ($d_{3,2}$) using the software ImageJ (Version 1.48, Wayne Rasband, National Institute of Health, Bethesda, Maryland, USA) [161]. One gram of fine starch powder was dispersed in 25 mL distilled water at 60 °C. Starch granule solution (40 µL) was pipetted onto a microscopic glass slide, followed by 20 µL iodine solution (0.2 g iodine in 100 mL aqueous solution) and a cover slide [286]. The slide was observed and imaged with Nikon H55S computerised microscope (bright field, phase contrast mode, a magnification of 10×100

times) [287]. Sudan black, a fluorescent dye, was used to stain milk lipids, and the CLSM images were viewed at 488 nm (excitation) and 515 nm (emission) [215].

3.32 PSD analysis

The particle size distribution (PSD) of starch granules in 60 °C aqueous dispersion was measured at 10% laser obscuration using the laser diffraction particle size analyser MasterSizer 3000 (Malvern Instruments Ltd., Malvern, Worcestershire, UK) equipped with a wet sample dispersion unit (Malvern Hydro MV, UK). The refractive indexes of starch and dispersant (water) were set as 1.54 and 1.33, respectively. The surface-area-based mean diameter ($d_{3,2}$) and volume-based mean diameter ($d_{4,3}$) were obtained in addition to particle size distributions [275]. The PSD of Milk fat globules in emulsion was also measured with MasterSizer 3000. The circulation motor was set at 2000 rpm, using ethanol as dispersant (10% laser obscuration.

3.33 Pancreatic-lipase-catalysed digestion of milk fats in dispersion or in starch gel

Simulated intestinal fluid (SIF) was composed of dipotassium phosphate (0.68 g in 100 mL aqueous solution), 150 mM sodium chloride, and 30 mM calcium chloride, which was maintained at 37ºC with continuous agitation at 95 rpm during digestion in a temperature-controlled multi-well stirrer platform [7]. The SIF was adjusted to pH 7.0 using 0.1 M NaOH prior to intestinal digestion.

An in vitro intestinal digestion model [288] was adapted to hydrolyse milk fat in both control and testing samples. A sample dispersion of 30 mL (containing 1 g milk fat), 10 mL SIF, pancreatic lipase (32 mg or 8,000 Lipase Unit), amyloglucosidase (Megazyme Australia, enzymatic activity 3.3 kU mL^{-1}, 100 µL or 330 U), and 200 mg bile extract were added to each pot to initiate the hydrolysis reaction. The reaction was carried out by constant agitation at 95 rpm and 37˚C. Aliquots of the digestion were taken at 0, 20, 40, 60, 90 and 120 min for free fatty acid content analysis.

3.34 Lipolysis and free fatty acid determination

The free fatty acid (FFA) content was assayed as the method in section 3.5.2.

3.35 Fatty acid methylation and gas chromatograph

In addition to FFA quantification, the free fatty acid profile was chromatographically analysed using the fatty acid methyl esters (FAME) method, with a gas chromatograph (GC) unit equipped with a flame ionization detector (FID) [54]. Milk lipid hydrolysates were lyophilized for two days and then resultant lipid powder (80 mg) was transferred to test tubes. Internal standard (C21:0 ester 5 mg/mL, 50 µL) was added to each tube. Heptane (450 µL) was transferred to each tube to dissolve the milk lipids, then 2.0 mL of ethanolic sodium hydroxide solution (0.5 M, anhydrous) was added to esterify the fatty acids. Fatty acid methylated esters were produced with a 15-min incubation in a 50˚C tube incubator. The produced esters were washed with heptane (1 mL) and distilled water (1 mL) by vortex (1 min). Subsequently, the mixture was centrifuged at 1500 *g* for 5 min. The esters in the supernatant were analysed by gas chromatography (GC) [289].

3.36 Kinetic analysis of lipolysis

The experimental data were fitted using a model described in the section of 3.5.2.

3.37 Statistical methods

The average of all results obtained were determined from triplicate measurements. Statistical analysis of results was carried out by one-way analysis of variance (ANOVA) with a Tukey test using Minitab 18 software (Minitab Inc., Chicago, USA). Significance was determined by a confidence level of 95%.

Chapter 4: Preparation and assessment of milk phospholipid-complexed antioxidant phytosomes with vitamin C and E: A comparison with liposomes

Chapter 4 has been submitted for publishing:

- Zhiguang Huang, Hui Zhao, Wenqiang Guan Jianfu Liu, Charles Brennan, Maneesha S. Mohan, Letitia Stipkovits, Haotian Zheng, Don Kulasiri. Preparation and assessment of milk phospholipid-complexed antioxidant phytosomes: with vitamin C and E. Food chemistry, 2019 (submitted).

Abstract: Phytosome assemblies have proved to be novel drug delivery systems. However, studies of phytosomes in food applications are scarce. The characteristics of milk phospholipid assemblies and their functionality in terms of *in vitro* digestibility and bioavailability of encapsulated nutrients (ascorbic acid and α-tocopherol) were studied. The phytosomes were prepared using ethanolic evaporation technique. Spectral analysis revealed that polar parts of phospholipids formed hydrogen bonds with ascorbic acid hydroxyl groups, further, incorporating ascorbic acid or α-tocopherol into the phospholipid assembly changed the chemical conformation of the complexes. Phospholipid-ascorbic acid phytosomes yielded an optimal complexing index of 98.52 ± 0.03% at a molar ratio of 1:1. Phytosomes exhibited good biocompatibility on intestinal epithelial cells. The cellular uptake of ascorbic acid was 29.06 ± 1.18% for phytosomes. It was higher than that for liposomes (24.14 ± 0.60%) and for ascorbic acid aqueous solution (1.17 ± 0.70%).

Keywords: Milk phospholipids; phytosome; liposome; encapsulation efficiency; *in vitro* digestion; cellular uptake

Pubchem CID: Bile salt (PubChem CID: 439520); L-ascorbic acid (PubChem CID: 54670067); α-tocopherol (PubChem CID: 2116); Lipase (PubChem CID: 135534490); Edetic acid (EDTA, Pubchem CID: 6049); Thiazolyl blue tetrazolium bromide (MTT, Pubchem CID: 64965)

4.1 Introduction

Milk contributes about one third of human dietary lipid intake. Milk phospholipids have been used as nutrient carriers since the early 2000s. Thompson [250] used milk phospholipids to

prepare three kinds of liposomes to encapsulate bioactive constituents. Since then, milk phospholipid-based liposomes and other encapsulated materials have been made to carry both lipophilic and hydrophilic components, for instance, curcumin [290], phenolic compounds [8], silybin [104], α-tocopherol [2], and ascorbic acid [3]. More recently, milk phospholipid liposomes have been manufactured as delivery systems, stabilizing the storage of tea polyphenols [8], solubilizing curcumin with a better efficiency than soy lecithin [9], and protecting lactoferrin from gastric disintegration [10]. In addition, Dynamic *in vitro* lipolysis and fatty acid titration assay have been used to understand the digestion of drug vesicles including phytosomes [291], and further, the vesicle property differences between lecithin and milk phospholipids have been made recently, and the latter was found to be more efficient in terms of encapsulation efficiency [9].

Both milk phytosomes and liposomes are derived from milk phospholipids, but they are distinct in several ways. Liposomes entrap bioactive compounds in either the core of phospholipid globule (hydrophilic encapsulates) or in the phospholipid bilayer (lipophilic encapsulates), forming closed vesicles (Ding et al, 2018; Lamba et al, 2018; Silva et al, 2018). Whereas, phytosomal complexes are constructed mainly by hydrogen bond between bioactive compounds and the polar heads of phospholipids. Phytosomes agglomerate into nano-size micelles (approximately 100 nm, slightly smaller than liposomes) when they are dispersed in aqueous phase. Hence they are integral conjugations, thereby more durable and efficient carriers than liposomes [89]. Commercial phytosomes, for instance, Silybin Phytosome™, Ginkgo Phytosome™, and Ginseng Phytosome™, use both synthetic and natural phospholipids to deliver bioactive constituents [292]. Phyto-phospholipid complexes are no longer limited to polyphenols either, and theoretically, the complexes can be used to deliver any active molecules [89].

Milk phospholipids are emerging biomaterials, however there are no phytosomes derived from them thus far. The present study aims to prepare two types of phytosomes by solvent evaporation, the types are: milk phospholipids-ascorbic acid phytosomes and milk phospholipids-α-tocopherol phytosomes. Parallel liposome based samples will be prepared as a control set for comparison in terms of nutrients encapsulation efficiency, assembly digestibility, and nutrients bioavailability using cellular uptake level as an indication. The formation of complexes was investigated by Fourier-transform infrared (FTIR) spectroscopy, ultraviolet (UV) spectroscopy, and differential scanning calorimetry (DSC).

4.2 Materials and methods

The materials and methods in the section of 4.2 can be referred back to the corresponding sections in Chapter 3, as illustrated in Table 4-1.

Table 4-1: Materials and methods for phytosomes.

Section 4.2	Materials and methods	As mentioned in the section of
4.2.1.	Materials	3.1
4.2.2.	Phospholipid isolation and determination	3.2
4.2.2.1.	Extraction of milk phospholipids	3.2.1
4.2.2.2.	Proximate chemical compositional analysis	3.2.2
4.2.3.	Preparation of liposomes and phytosomes	3.3
4.2.3.1.	Preparation of liposomes	3.3.1
4.2.3.2.	Preparation of phytosomes	3.3.2
4.2.3.3.	CI of phytosomes	3.3.3
4.2.3.4.	EE of liposomes	3.3.4
4.2.4.	Verification of phytosomal complexing	3.4
4.2.4.1.	FTIR	3.4.1
4.2.4.2.	UV spectrum analysis	3.4.2
4.2.4.3.	DSC analysis	3.4.2
4.2.5.	*In vitro* digestion	3.5
4.2.5.1.	Simulated intestinal digestion	3.5.1
4.2.5.2.	Release of ascorbic acid and free fatty acids	3.5.2
4.2.6.	*In vitro* absorption and cytotoxicity	3.6
4.2.6.1.	Cell culture	3.6.1
4.2.6.2.	The cellular uptake of ascorbic acid	3.6.2
4.2.6.3.	Cytotoxicity	3.6.3
4.2.7.	Statistical methods	3.37

4.3 Results and discussion

4.3.1 Isolation and fractionation

The total phospholipid content of the original phospholipid enriched milk powder was 12.80 ± 1.30% (w/w). The phospholipid content in the phospholipid extract was 91.14 ± 1.56% (w/w). The relative contents of proteins and triacylglycerols were 4.50 ± 1.18% and 4.35 ± 1.71% (w/w), respectively. The purity of phospholipid in the phospholipid extract samples obtained in this study was higher than those of Fonterra Phospholac 600 and Phospholac 700 [31], and Arla Lacprodan PL-75, which were 69.8%, 61.55%, and 75%, respectively. The increased purity of milk phospholipids observed in this study is likely due to the thrice defatting by acetone.

Phytosomes can be manufactured in several ways, for instance, anti-solvent precipitation or evaporation, and anhydrous co-solvent lyophilisation [89]. Traditionally, phytosomes were usually prepared in an aprotic solvent such as dioxane, acetone, methylene chloride, or ethyl acetate, by conjugating phospholipids with active components at molecular ratios of 1:1 – 3:1. More recently, protonic solvents, such as ethanol and methanol, have been successfully used to prepare phytosomes. For example, silybin phytosomes were successfully formed in ethanol [89]. In this study, the ethanolic evaporation technique was used to prepare phytosomes because of its food-compatibility [89, 293].

In contrast, liposomes have been manufactured by several methods including reverse phase evaporation, hydration lipid film, spray drying, and lyophilisation. In the present study, sonication-assisted thermal hydration was adapted for liposomal manufacture due to its simplicity.

4.3.2 Liposomal EE and phytosomal CI

EE of liposomes

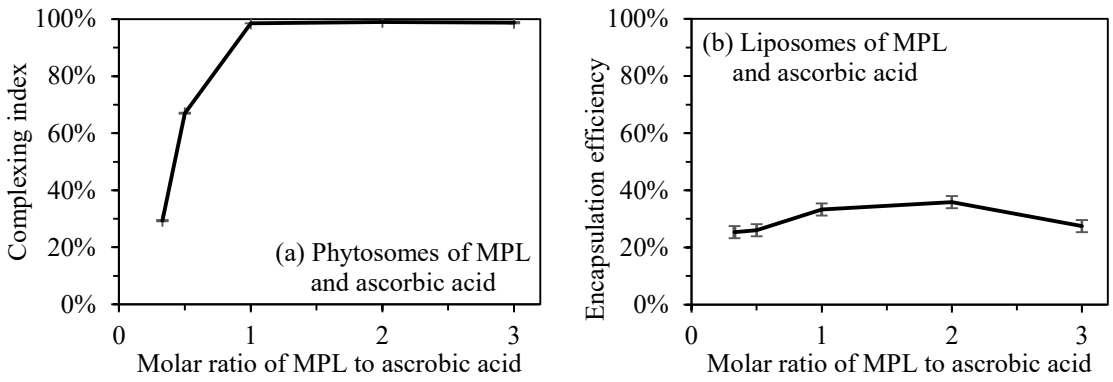
The molar ratio of liposomes was optimized at 2:1 (Figure 4-1b), yielding an efficiency of 35.88 ± 0.14%, comparable to a recent report on liposomes [253]. The encapsulation efficiency of liposomes is considered to be dependent on several factors, including both the constituents and manufacture methods. For example, in another study on supercritical fluid-made liposomes, the encapsulation factors for olive pomace extract and linalool were 58% and 55%, respectively [294]. Concerning the molar ratio of phospholipids to bioactive molecules, two recent reports prepared phenolic compounds and conjugated with linoleic acid with molar proportions at 1:1 and 2:1, respectively [253, 295].

CI of phytosomes

As illustrated in Figure 4-1a, milk phospholipids conjugated with ascorbic acid (176.12 g mol^{-1}) at different molar ratios, ranging from 0.33:1 to 3:1. The optimal CI of phytosomes was 98.52 ± 0.03% at molar ratio of 1:1, which was much higher than the encapsulation efficiency of liposomes (35.88 ± 0.14% at a molar ratio of 2:1). The complexing indexes of phytosomes plateaued after reaching the optimal efficiency. Whereas for α-tocopherol (430.71 g mol^{-1}) complexation phytosomes had their highest complexing factor at the stoichiometric ratio of 3:1 (Figure 4-1c), probably due to difference of molecular weight and bonding affinity to phospholipids.

In general, phytosomes are constructed of a phospholipid-bioactive compound conjugation in a molar proportion of 0.5 – 2.0, with an optimal index at the molar ratio of 1:1 in many cases [89]. In another report on phospholipid-oleanolic acid phytosomes, the molar ratio of 1:1 led to the best loading efficiency. However, the efficiency at molar ratios of 2:1 and 3:1 did not differ from those complexes (oleanolic acid phytosomes) with a molar ratio of 1:1 [296]. The optimal complexing molar ratio of phospholipids to bioactive constituents is dependent on both the carrier and the encapsulated bioactive molecules. For example, in silymarin-phospholipid phytosomes, the optimum efficiency was achieved at a molar ratio of 5:1 [297]. Furthermore, in oxymatrine phytosomes, the stoichiometric ratio of 3:1 generated a higher CI than those at other ratios [298]. In a drug phytosome, the molar ratio was optimized at 3:1 to achieve the highest entrapment of berberine hydrochloride, nevertheless, there was no distinctive difference in the efficiency when this ratio was 4:1 [299]. In the present study, α-tocopherol phytosomes yielded the highest efficiency at molar ratio 3:1.

Phytosomes exhibited higher binding efficiency than liposomes, consistent with a previous report on rutin encapsulation, where phytosomes led to an entrapment efficiency of 24 ± 2%, compared with that of liposomes (17 ± 10%) [252]. In another system (egg yolk phosphatidylcholine therapies), the CI of phytosomes was 49 ± 3%, almost double that of liposomes [300]. The observed difference in the CI and EE between phytosomes and liposomes could be attributed to the hydrogen bonding between bioactive compounds and phospholipids, providing association sites at both the interior and exterior membranes of the lipid vesicles [301]. In contrast, liposomes can only entrap bioactive molecules either in the core (lipophilic) or within the lipid bilayer (hydrophilic) [89]. Thus, phytosomes frequently exhibit high complexing indexes, as shown in two previous studies (98.4% and 97.2%, respectively) [297, 302].



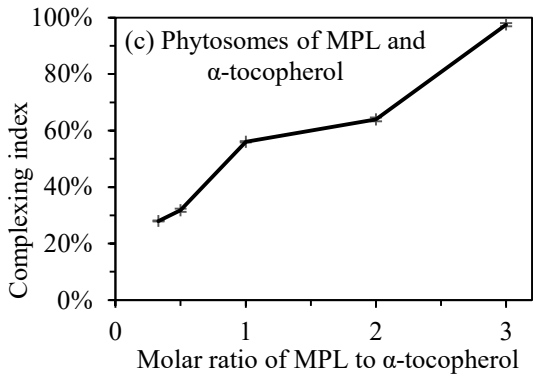


Figure 4-1: Encapsulation efficiency of MPL-derived phytosomes and liposomes.

Notes: The complexing index (CI) of milk phospholipids (MPL)-ascorbic acid phytosomes (a); the encapsulation efficiency (EE) of MPL-ascorbic acid liposomes (b), and the complexing index of phytosomes between MPL and α-tocopherol (c).

4.3.3 Spectroscopy study

Fourier-transform infrared spectroscopy

Since liposomes displayed significant lower encapsulation efficiency ($p < 0.05$) than phytosomes, FTIR spectral analysis only focused on phytosomes, their constituents, and the mixture, as illustrated in Figure 4-2. When the spectrums of ascorbic acid (Figure 4-2c) and the mixture of constituents (Figure 4-2d) were compared, it was worthy to note that the characteristic bands of ascorbic acid in Figure 4-2c (3525 – 3219 cm^{-1} and 1672 – 756 cm^{-1}) were also visible in the spectrum of the mixture in Figure 4-2d, which suggests that there was no formation of new chemical bonds during mixing, consistent with a previous study by Telange *et al* [93]. At the same time, the aliphatic groups of phospholipids (2920 cm^{-1}, 2850 cm^{-1}, and 1741 cm^{-1} in Figure 4-2a) were also inherited in the mixture spectrum in Figure 4-2d, whereas others were diluted and invisible, in line with a previous report by Zhang *et al* [255].

When the spectrums of milk phospholipids (Figure 4-2a) and phytosomes (Figure 4-2b) were compared, it was found that in the phytosomal conformation the peaks from the phospholipid bonds P=O (1236 cm^{-1}), P–O–C (1092 cm^{-1}), and [– $N^{+}(CH_2)_3$] (970 cm^{-1}) of the choline residues shifted to 1207 cm^{-1}, 1061 cm^{-1}, and 974^{-1} cm, respectively. Whereas the aliphatic signals in Figure 4-2a including carboxyl ester bond C=O (1741 cm^{-1}), hydroxyl bond (3390 cm^{-1}), and methyl and methylene groups of fatty acids (2920 cm^{-1}, 2850 cm^{-1}, and 1468 cm^{-1}) were unchanged in both the phytosomes (Figure 4-2b) and the mixture of constituents (Figure 4-2d). This clearly indicates that the polar heads of milk phospholipids in phytosomes interact

with hydroxyl groups of ascorbic acid, resulting in the replacement of the major bonds in the phosphatidyl residues in phospholipids, whereas the non-polar heads of fatty acids including methyl, methylene groups and carboxyl ester bond C=O exhibited no chemical interaction.

In contrast to the collective effect of the mixture spectrum (Figure 4-2d) from its constituents, the spectrum of phytosomes (Figure 4-2b) revealed synthetic effect on the constituents. For instance, characteristic signals (3410 cm^{-1} and 1672 cm^{-1} for O–H and C=O groups, respectively) in ascorbic acid (Figure 4-2c) merged into new peaks at 3390 cm^{-1} and 1741 cm^{-1} in Figure 4-2b, respectively. A recent study, evaluating the hydroxyl stretch (O–H, 3377 cm^{-1}) of lipophilic drug dihydroartemisinin (DHA), also illustrated such broadening complexing with phospholipids [303]. The similar change has been observed in apigenin phytosomes, in which a peak of phenolic O–H group moved to 3338 cm^{-1} from 3272 cm^{-1} [93]. Further evidence in celastrol phytosomes is provided by Freaga *et al* [92], where celastrol peak at 1689 cm^{-1} (C=O stretching) shifted to 1738 cm^{-1} after formation of phytosomes.

Similar observations have been reported on spectral change during formation of soy phosphatidylcholine phytosomes has been reported, where the P=O peak (1251 cm^{-1}) went to 1240 cm^{-1} [91]. In addition, the results of Figure 4-2c are congruous with a recent record, in which the P=O peak at 1237 cm^{-1} (from the polar head group) disappeared in the spectra of silymarin phytosomes [297]. Further evidence is provided by Telange *et al.* [93], where both P=O and P–O–C peaks were altered in phospholipid complexes, as a result of intermolecular interactions and conformation of complexes between apigenin and phospholipid molecules. In addition, the peak at 1645 cm^{-1} (C=C of milk phospholipids in Figure 4-2a) shifted to 1684 cm^{-1} in phytosomes in Figure 4-2b, and one possible explanation is that, on formation of phytosomes, ascorbic acid interacts with the polar heads of phospholipids molecules, while fatty acid chains in *sn*-1/2 positions may rotate freely to wrap the polar heads of ascorbic acid. Thus their wavenumbers in FTIR spectrum were rearranged, in support of previous reports [304, 305]. Based on the previous evidences, and the results reported here, we conclude that polar parts of phospholipids formed hydrogen bonds with ascorbic acid hydroxyl groups.

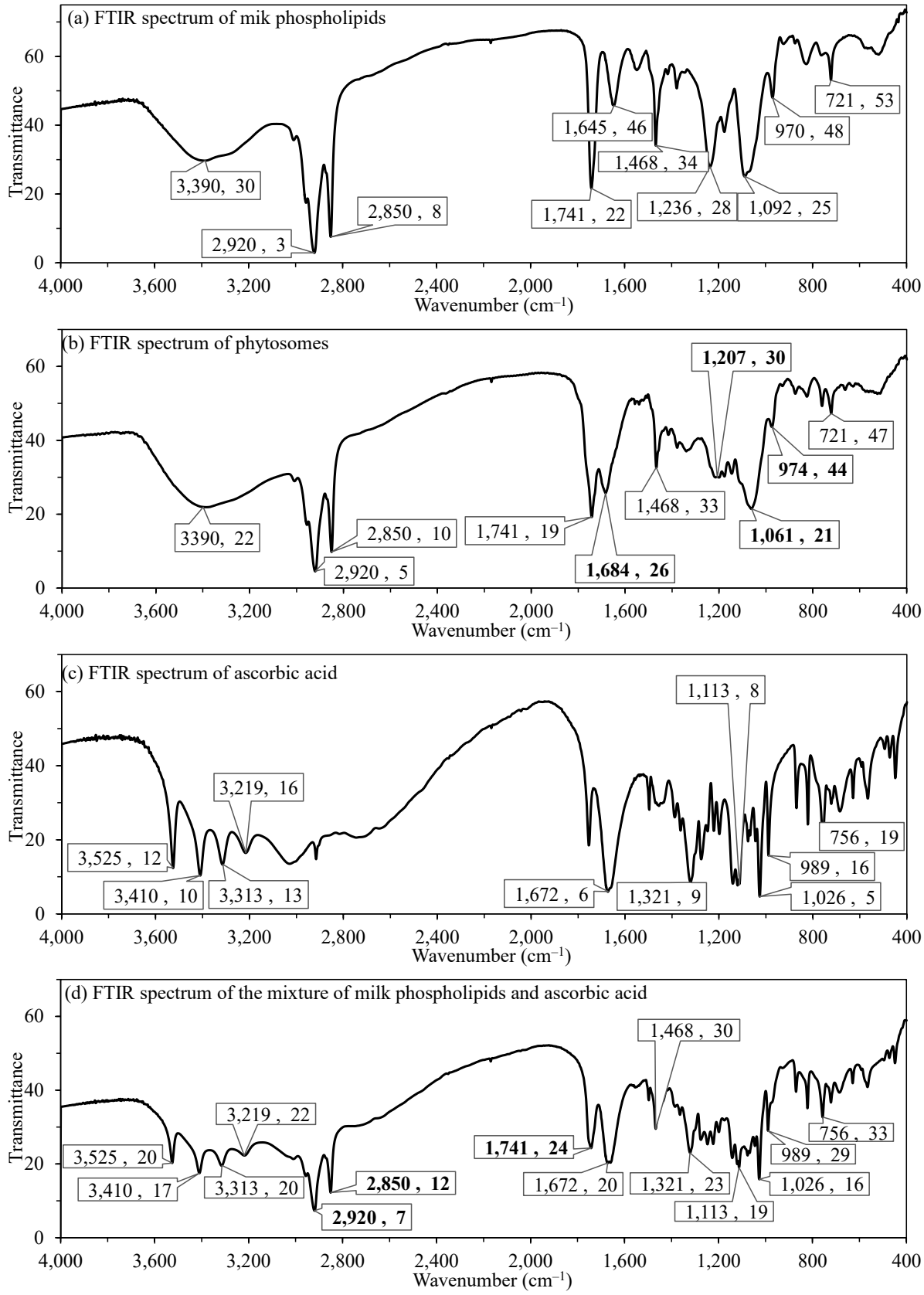


Figure 4-2: FTIR spectrum of MPL and their constituents.

Notes: Milk phospholipids (MPL) (a); MPL-ascorbic acid phytosomes (b); ascorbic acid (c); and the mixture of MPL and ascorbic acid (d).

UV spectra

The UV spectrums of milk phospholipids, ascorbic acid, their mixture, and phytosomes exhibited analogous profiles, but with significant differences in peak wavelengths ($p < 0.05$), as shown in Figure 4-3. Ascorbic acid displayed its main peak at 255.5 nm (Figure 4-3c), while the milk phospholipids exhibited a major peak at 209.5 nm (Figure 4-3a).

When milk phospholipids and ascorbic acid were mixed together in ethanolic dispersion, both major peaks (209.5 nm for phospholipids and 255.5 nm for ascorbic acid (Figure 4-3d), respectively) were present in the resultant spectrum. However, when the two components were incorporated as phytosomes (Figure 4-3b), the major peaks shifted to 257 nm and two adjacent, minor peaks at 203 nm and 221 nm, indicating a change in the chemical conformation of the complexes. Similar spectra with multi-peaks have been reported for an aqueous dispersion of silybin phytosomes, in which both a principle maximum and a weak shoulder were identified at 285 and 325 nm, respectively. The spectral change was attributed to the molecular interactions between constituents in the form of hydrogen bonding [254]. Further, the UV spectrum of egg phosphatidylcholine-derived vesicles displayed a maximum peak at 254.5 nm in aqueous solution and 259.7 nm in aqueous system and carbon tetracholoride, respectively [306], which is very close to the major peak of 257 nm in the present phytosomes.

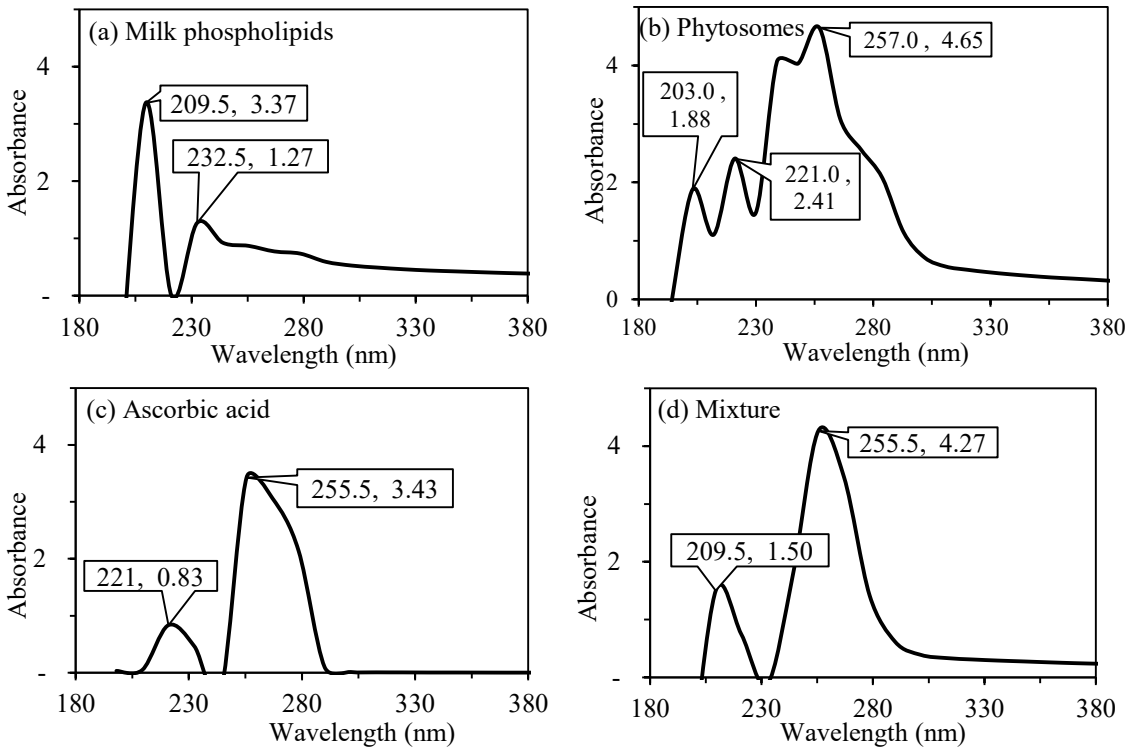


Figure 4-3: UV spectra of MPL phytosomes and their constituents.

Notes: Milk phospholipids (MPL) (a); MPL-ascorbic acid phytosomes (b); ascorbic acid (c); and the mixture of MPL and ascorbic acid (d).

DSC analysis

The interactions between milk phospholipids and the encapsulated constituents in phytosomes can be investigated further using DSC, by comparing the transition temperature, appearance of new peaks, disappearance of original peaks, melting points, and changes in the relative signal of DSC spectroscopy [89]. As shown in Figure 4-4c, the DSC thermogram of ascorbic acid exhibited a major endothermic peak at 193.85˚C, which corresponds to its crystalline melting point. Phospholipids (Figure 4-4a) demonstrated an obvious sharp endothermic peak at 47.82˚C, two following blunt peaks at 85.64˚C and 128.15˚C, and further two successive peaks at 227.35˚C and 262.17˚C, similar to the DSC results on quercetin phytosomes documented by Zhang *et al.* [255] in which peaks of heat-induced polar-head motion, crystal-liquid phase transition, and non-polar hydrocarbon tail of phospholipid melting were observed at 143.6˚C, 228.3˚C, and 239.4˚C, respectively. In addition, Ma *et al.* [305] recorded one exothermic peak in mangiferin phytosomes at 251.25˚C (crystal-liquid transition point). With regards to the mixture (Figure 4-4d), the melting points of both the phospholipids (47.82˚C) and ascorbic acid (193.85˚C) shifted slightly to 48.53˚C and 193.29˚C, respectively. Furthermore, the thermal transition at 181.16˚C in Figure 4-4d may be the crystal-liquid transitional point of phospholipids. In addition, there were distinct peaks at 195.67˚C (endothermic) and 206.02˚C (exothermic) in Figure 4-4d, which may be due to the disintegration of constituents (around 195.67˚C) and further reactions among the newly produced intermediates and the original constituents (approximately 206.02˚C). The melted ascorbic acid (antioxidant) and phospholipids (both antioxidant and pro-oxidant) could interact in several ways, consequently resulting in the multiple high-intensity endothermic (concave peaks for breakdown or endothermic reactions) and exothermic (convex peaks for exothermic reactions) peaks, as presented in Figure 4-4d.

On the other hand, the DSC spectrum of phytosomes (Figure 4-4b) was totally different from that of the mixture (Figure 4-4d). The phospholipid melting point of 48.53˚C in the mixture (Figure 4-4d) shifted to a minor endothermic peak at 52.44˚C. Furthermore, the major endothermic peak in the spectra of phytosomes (180.96˚C in Figure 4-4b) may be the result of the phase transition of crystalline phytosomes or due to the dissociation of phospholipid-ascorbic acid interactive forces (hydrogen bonds and van der Waals forces). This is in support of a previous DSC study by Tan *et al.* [307]. In addition, there are further endothermic peaks

at 244.07˚C and 281.56˚C in Figure 4-4b. It is possible these indicate a series of splitting of the milk phospholipids or further dissociation of the phytosome complexes, as reported by Zhang *et al.* [255]. Furthermore, those endothermic peaks could equally be due to the melting, isomerisation or crystal change of the phytosomes, as proposed in another study on phytosomes [308]. Altogether, the DSC spectra observed in this present study are in agreement with DSC data on phytosomes that has been published [103].

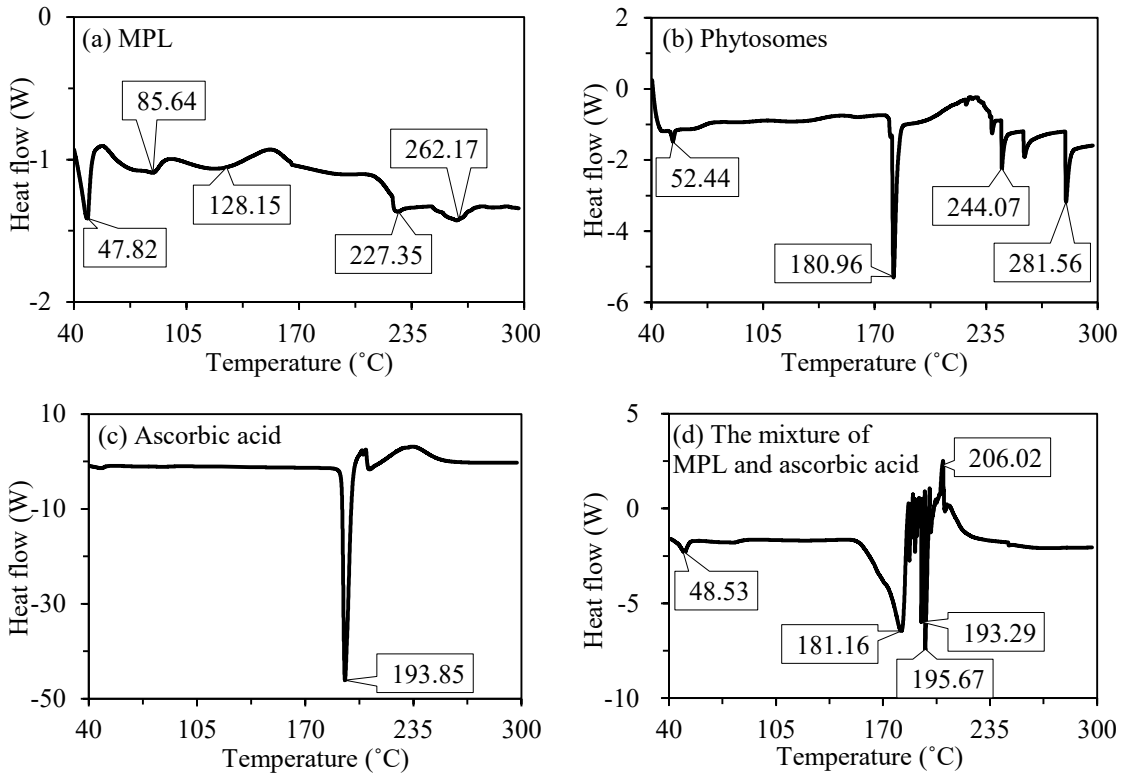


Figure 4-4: DSC thermograms of MPL and their constituents.

Notes: Milk phospholipids (MPL) (a); MPL-ascorbic acid phytosomes (b); ascorbic acid (c); and the mixture of MPL and ascorbic acid (d).

4.3.4 *In vitro* digestion and absorption

In vitro digestion

Gastric pH and salts can alter the size the structure of particles, which further affect the intestinal digestion. However, gastric digestion contributes to only a minor proportion (1.5 – 8%) of the total phospholipid hydrolysis [293] and takes place at pH 1.5 – 3, which is incompatible with the present titration quantification since both gastric acid and free fatty acids consume titrant. Therefore, only intestinal digestion was surveyed this study, as also commonly performed by recent reports on lipid digestion [309-311]. As illustrated in Figure 4-5, the release velocities of fatty acids from phytosomes and liposomes were significantly

different ($p < 0.05$). Disintegration rate is often used to determine the stability of encapsulation vesicles [10]. In the present study, liposomes exhibited a higher degradation rate than phytosomes, indicating that phytosomes are comparably more stable matrices. In addition, the slow disintegration of phytosomes may be due to the hydrogen bonding between constituents, thus it takes a comparably longer time to be detached from the carrier than those in complexed matrices [300]. In another study, the formation of stable H-bonding in phytosomes has been attributed to a better structural integrity, bioavailability, and absorption in phytosomes compared with liposomes [301]. Phytosomes have also been reported to not only increase the area under the concentration-time curve (AUC) but also prolong the half-life of the particles [312].

Under the catalysis of pancreatic lipase, the fatty acid release from phytosomes and liposomes followed by first-order kinetics, with reaction rate constants of 0.016 ± 0.001 min^{-1} and 0.034 ± 0.007 min^{-1}, respectively. The half digestion time for phytosomes and liposomes is 44.71 ± 1.14 min (R^2=0.97) and 20.15 ± 0.08 min (R^2=0.93), respectively. The reaction rate constant of phytosomal degradation is lower than that of liposomes, thus the phytosomes disintegrated at a slower rate than the liposomes. This difference may be due to the particle structural discrepancy and related difference in interfacial properties between phytosomes and liposomes, as reported by Manici *et al.* [300].

As shown in Figure 4-5, the release of both ascorbic acid and fatty acids demonstrated a similar trend, indicating the synchronous liberation of ascorbic acid and fatty acids with the disintegration of the particles.

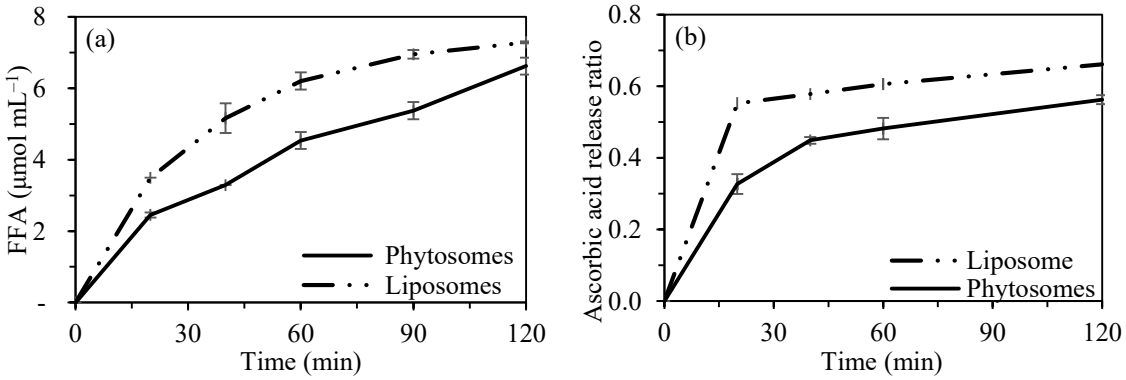


Figure 4-5: FFA and ascorbic acid release during intestinal hydrolysis.

Notes: FFA (a) and ascorbic acid (b).

Cellular uptake

The cellular uptake of ascorbic acid from phytosomes (29.06 ± 1.18%) was greater than that from liposomes (24.14 ± 0.60%) or from ascorbic acid aqueous solution (1.17 ± 0.70%), consistent with a previous report where phytosomes exhibited a higher propensity across cell membranes and resulted in a massive cellular uptake [254]. Further, bioflavonoids in phytosomal complexes (US5043323A-1991) have been found to be more bioavailable than uncomplexed flavonoids using a mouse model. The greater degree of phytosomal cellular uptake is likely because the membrane permeability and oil-water partition coefficients of phytosomes are remarkably enhanced. Therefore, bioactive constituents in phytosomes are more readily absorbed than those in liposomal matrices and aqueous solution [89]. Phytosomes may be absorbed from the intestinal tract through enterocyte-based transport, and bioactive compounds then transport to the systemic circulation via the intestinal lymphatic system [90].

MTT assay

As illustrated in Figure 4-6, both phytosomes and liposomes showed no detrimental effect on the viability of IEC-6 cells, which demonstrated their safety with intestinal cells. The slight increase (insignificant, $p < 0.05$) in cell viability may be due to the antioxidant activities of ascorbic acid. This biocompatibility was consistent with a mice-model study on the cytotoxicity of phosphatidylcholine [313]. Also, phosphatidylserine exhibited no adverse effects via oral administration in an *in vivo* model [314]. Being biodegradable, liposomes in aqueous dispersion have been used to deliver both lipophilic and hydrophilic bioactive molecules since the 1960s. Therefore, both phytosomes and lipsomes can be regarded as biocompatible and effective to integrate into food systems.

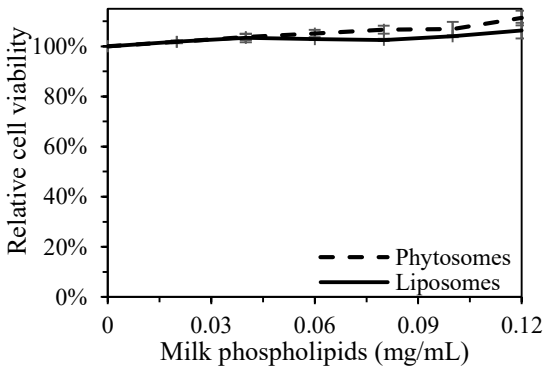


Figure 4-6: Relative cell viability in MPL dispersion.

Notes: Data was expressed as% of untreated, control samples at various concentrations.

4.4 Conclusion

In this study, phospholipids were isolated and refined from milk products and prepared into both phytosome and liposome particles. Ascorbic acid phytosomes exhibited higher encapsulation efficiencies (optimal complexing index 98.52 ± 0.03% at molar ratio 1:1) than liposomes. The spectral analyses of phytosomes suggest that ascorbic acid associates through hydrogen bonding at the polar heads of phospholipids, inducing the rearrangement of P=O and P – O – C stretches of the polar heads of milk phospholipids. In addition, *in vitro* digestion showed that the cleavage of both fatty acids and ascorbic acid from phytosomes were slower than those from liposomes. Cellular uptake studies displayed that phytosomes were more readily absorbed than liposomes. Both matrices demonstrated biocompatibility, without compromising on safety. In summary, these results reveal that milk phospholipid-based phytosomes had greater *in vitro* digestion stability than liposomes, and thus show potential to be developed as nutrients for functional foods or infant formulas.

Chapter 5: Milk phospholipids antioxidant activity and digestibility: kinetics of fatty acids and choline release

Chapter 5 has been submitted for publication:

- Zhiguang Huang, Hui Zhao, Wenqiang Guan Jianfu Liu, Charles Brennan, Maneesha S. Mohan, Letitia Stipkovits, Don Kulasiri. Milk phospholipids antioxidant activity and *in vitro* digestion: kinetics of fatty acids and choline releases. Journal of Functional Foods, 2019 (submitted).

Abstract: Milk phospholipids have been used as functional ingredients in foods recently. Thus, there have been no reports on how polarity of lipids affects their digestibility. This study aims to isolate milk phospholipids from dairy products, characterize them chromatographically, and assess their digestibility and antioxidant activities *in vitro*. The results revealed that their lipolysis reaction rate constants were significantly different ($p < 0.05$) between milk phospholipids and triacylglycerols, and thus the degradation of both lipids followed first-order reaction kinetics. Furthermore, the cellular uptake of milk phospholipids was determined with a HT-29 cell model, and they were found not to be absorbed intact during intestinal digestion. Milk phospholipids exhibited significant antioxidant activity *in vitro*, while their cellular antioxidant activity (CAA) was very limited. The results of this study provide useful information for the design of milk phospholipid-based carrier systems.

Keywords: *In vitro* phospholipid digestion; Choline phospholipid digestion; Fatty acid release; Cellular uptake; Antioxidant activity

5.1 Introduction

Phospholipids are important ingredients in milk. They serve as functional nutrients with both technological importance and nutritional relevance [13]. For instance, milk phospholipids have been used as vesicle materials since the early 2000s. Thompson [6] used milk phospholipids to prepare three kinds of liposomes to carry bioactive molecules. Since then, milk phospholipid-based liposomes have been prepared to deliver ascorbic acid [3] and lactoferrin [7]. More recently, milk phospholipid liposomes have been applied to improve the storage stability of encapsulates [8], the encapsulation efficiency [9, 10], and the delivery system for anticancer drugs [11], with milk phospholipids showing higher efficiency compared with soy lecithin [10]. Phospholipids have been used to prepare antioxidant

phytosomes [300] and to modulate the release of antioxidant from phospholipid vesicles [252]. The digestion kinetics of phospholipids control the bioavailability of encapsulated bioactive compounds and impact on their release [315]. Native milk phospholipids are mainly located in the bilayer membrane of tri-layered globules in colloidal structure, which may affect the release of bioactive compounds along the gastrointestinal tract [39].

In addition to the vesicle properties, milk phospholipids have been refined as functional ingredients. Phospholac 500, 600, 700 and Gangolac 600 have purities of 33.9%, 69.8%, 61.6%, and 14.5%, respectively [31, 55], while Lacprodan® MFGM 10 and Lacprodan® PL 20 (purity 20%) have been produced for functional food ingredients [48] and infant formulas [56]. Lipidex, a derivative from β-serum powder, contains 5 – 7% phospholipids and 26.6% fat in total [59], and bovine milk sphingomyelin has a purity of 99% [38]. In addition, Lipamine M20, which has been manufactured with membrane separation, comprises of 20% of sphingomyelin, ceramides, and ganglioside [60]. Ethanol is the most commonly used solvent to extract milk phospholipids, for instance, hot alcohol (70%) extraction at 70˚C rendered 45.8% purity, starting from whey protein phospholipid concentrate [47]. Supercritical carbon dioxide extraction can be used to enrich polar lipids by removal of neutral lipids [316]. In a laboratory up-scaling test on buttermilk powder substrate, a pilot unit integrated enzymatic proteolysis, ultrafiltration, and super-critical fluid extraction, yielding a dry-matter purity of 56.24 ± 0.07% [49, 317].

There have been a few reports on the digestibility of highly-purified milk phospholipids. For example, phospholipase A_2 is the enzyme responsible for phospholipid hydrolysis, but milk phospholipid liposomes disintegrate slower than soy lecithin liposomes [10, 39]. There have also been no reports on how polarity impacts lipid digestion so far. Therefore, the present study aims to extract and refine phospholipids from dairy products, to characterize them chromatographically, and to evaluate their stability in simulated intestinal fluids and cellular update on HT-29 cells, and *in vitro* antioxidant activity.

5.2 Materials and methods

The materials and methods in the section of 5.2 can be referred back to the corresponding sections in Chapter 3, as illustrated in Table 5-1.

Table 5-1: Materials and methods for MPL digestibility and antioxidant activity

Section 5.2	**Materials and methods of milk phospholipids (MPL)**	**As mentioned in the section of**

5.2.1.	Materials	3.7
5.2.2.	Extraction and purification of milk phospholipids	3.8
5.2.2.1.	Milk phospholipids extraction	3.8.1
5.2.2.2.	Solid phase extraction (SPE)	3.8.2
5.2.3.	Chemical analysis	3.9
5.2.3.1.	Proximate chemical compositional analysis	3.9.1
5.2.3.2.	Thin layer chromatography (TLC)	3.9.2
5.2.4.	*In vitro* digestion	3.10
5.2.4.1.	Simulated intestinal digestion catalysed by pancreatic lipase	3.10.1
5.2.4.2.	Release kinetics of FFA from milk phospholipids	3.10.2
5.2.4.3.	Choline release from milk phospholipids	3.10.3
5.2.5.	Absorption and cytotoxicity	3.11
5.2.5.1.	Cell culture	3.11.1
5.2.5.2.	Cellular uptake of phospholipids	3.11.2
5.2.5.3.	Anti-proliferation activity	3.11.3
5.2.6.	Antioxidant activity	3.12
5.2.6.1.	DPPH radical scavenging activity	3.12.1
5.2.6.2.	ABTS radical scavenging activity	3.12.2
5.2.6.3.	Hydroxyl radical scavenging activity	3.12.3
5.2.6.4.	Thiobarbituric acid reactive substance (TBARS) inhibition method	3.12.4
5.2.6.5.	Cellular antioxidant activity (CAA)	3.12.5
5.2.7.	Statistical method	3.37

5.3 Results and discussion

5.3.1 Proximate nutritional analysis

The total phospholipid content of milk polar-lipid extract was 91.14 ± 1.56% (w/w) and 12.80 ± 1.30% (w/w) after and before extraction, respectively. The relative protein and triacylglycerol contents were 4.50 ± 1.18% and 4.35 ± 1.71%, respectively. Compared to previous reports [31, 318], the increased purity of milk phospholipids observed in this study was due to the repeated defatting using solvents at laboratory.

5.3.2 Chromatographic analysis of milk phospholipids

The phosphatidylcholine (PC) band was first located by aligning its R_f with that of standard PC, then the others (*i.e.* SM, PS, PE, PI) were be determined by their ranking of R_f. As illustrated in Figure 5-1, the bands appeared in an ascending order sphingomyelin (SM, R_f=0.21 ± 0.00), phosphatidylserine (PS, R_f=0.34 ± 0.00), PC (R_f=0.62 ± 0.02), phosphatidylethanolamine (PE, R_f=0.88 ± 0.01), and phosphatidylinositol (PI, R_f=0.95 ± 0.04). The comparative area of each spot were measured with ImageJ Software (National Institutes of Health, Bethesda, MD, USA), resulting in a relative content of phospholipid

species (SM 12.5 ± 1.8%, PS 17.7 ± 0.5%, PC 36.9 ± 2.9%, PE 19.6 ± 5.3%, PI 9.5 ± 1.0%). The current relative content of the phospholipids which were obtained from the samples was comparable to a recent report using HPLC evaporative light scattering detector (ELSD) assay [319]. A typical, relative composition of bovine milk phospholipids was also analysed with P^{31} NMR in a previous report [320], with similar results to that of the TLC reported here except for the content of PE. The discrepancy may be due to the differences in both samples and assays.

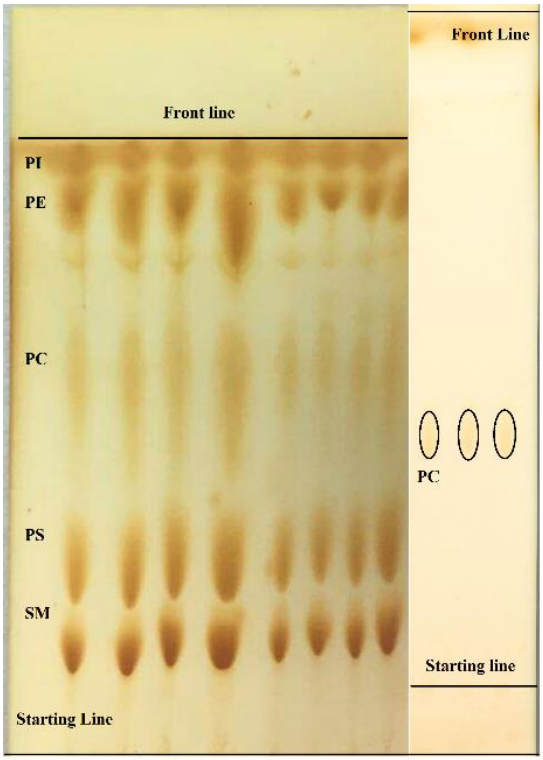


Figure 5-1: TLC image of MPL and PC.

Notes: The chromatography of milk phospholipids (MPL) and standard phosphatidylcholine (PC, right), and the relative position was determined by aligning the R_f value of each band with that of the reference.

5.3.3 Milk phospholipid digestion and cellular uptake

FFA from milk phospholipids and triacylglycerols

Gastric digestion contributes only a minor proportion (1.5 – 8%) of the total phospholipid hydrolysis [293]. Normally, gastric digestion occurs at pH 1.5 – 3, which was incompatible with the present titration quantification since both gastric acid and FFA consume titrant. AACC 02.31.01 method [182] was used to titrimetrically quantify FFA. The titratable acidity

of lipophilic solution can be best detected with ethanolic potassium hydroxide in the presence of diethyl ether [321], as also adapted in an AOAC method [256].

As illustrated in Figure 5-2a, both milk phospholipids and triacylglycerols were digested following first-order kinetics, but with different reaction velocity and degrees. In the first 20 min, approximately 56% of fatty acids were released from milk polar lipids, much higher than that of neutral lipids. Following 2 h of intestinal digestion, the hydrolysis degrees were 91.60 ± 4.04% and 56.71 ± 3.51% for phospholipids and triacylglycerols, respectively. Their breakdown rate constants were 0.007 min^{-1} and 0.016 min^{-1}, corresponding to a half-digestion time of 6.35 min and 43.86 min, respectively. The results are comparable to a previous report on lipolysis kinetics by type II lipase with L-α-phosphatidylcholine (Sigma-Aldrich, 1%, w/w) [322].

The lipolysis fits first-order kinetics (R^2=0.98 and 0.99 for phospholipids and triacylglycerols, respectively), as also reported on hydrolysis of palm, rapeseed, and linseed oil [323] and caroteniod enriched olive oil in phosphatidylcholine emulsion [322]. Milk phospholipids exhibited a higher digestion velocity and hydrolysis degree than triacylglycerols, indicating the polarity influence on the reaction rate constant. The difference in the kinetics might be due to several factors, such as the difference in lipase catalysis and interfacial properties between phospholipids and triacylglycerols.

In humans, pancreatic phospholipase A_2 (EC 3.1.1.4) can act upon the *sn-2* position of phospholipids, resulting in lysophospholipids and fatty acids [96]. The fatty acid group of lysophospholipids can be further cleaved by lysophospholipase (EC 3.1.1.5) [324]. Furthermore, the pancreatic lysophospholipase of human beings is most likely a non-specific phospholipase, that is, carboxyl ester hydrolase (CEH, EC 3.1.1.1, wide substrate specificity) [98]. In addition, CEH can also attack intact phospholipids [325], whereas, triacylglycerol lipase (EC 3.1.1.3) acts on triacyglycerols concomitantly with co-lipase, in the presence of bile salt [323]. In brief, different enzymes are involved in the hydrolysis of triacyglycerols and phospholipids during intestinal digestion, thereby their reaction rate constants differ from each other.

Lipopan F fungal lipase (EC 3.1.1.X, wide substrate specificity) was used to break down phospholipids and triacylglycerols in the present study. As illustrated in Figure 5-2b, the lipolysis reaction rate constants under the fungal lipase catalysis were 0.002 ± 0.001 min^{-1} and 0.007 ± 0.001 min^{-1} for phospholipids and triacylglycerols, respectively, corresponding to

half digestion time of 289.80 ± 21.52 min (R^2=0.96) and 97.69 ± 8.88 min (R^2=0.97). Since phospholipid hydrolysis was faster than that of triacylglycerols in both specific lipase and non-specific lipase (Lipopan F), there must be factors other than lipases that contributed the reaction rate difference of the two lipolysis reactions.

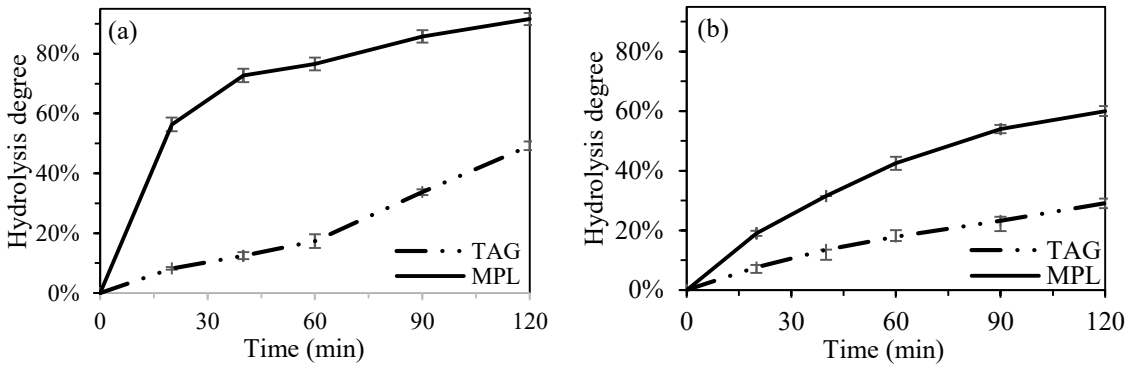


Figure 5-2: Milk lipolysis by two kinds of lipases.

Notes: Pancreatic lipase (a) and fungal lipase Lipopan F (b); MPL: milk phospholipids, TAG: triacylglycerols.

Lipolysis occurs by interfacial catalysis, which involves two equilibrium processes. Firstly, the adsorption of the lipase to the lipo-aqueous interface, followed by the subseqent conformation of the acyl-lipase complex intermediate [325]. The lipase must penetrate the interface to act, therefore the interfacial properties of substrates (*e.g.* in the form of micelles, emulsions, liposomes, or vesicles), have a direct impact on lipolysis kinetics [326]. In additon to colloidal structure, the lipolysis reaction rate constant is also dependent on lipid composition. For instance, short chain fatty acids hydrolyse faster than long chain fatty acids, and whereas milk fat breaks down faster than soy and fish oil [315]. Milk phospholipids and triacyglycerols have similar fatty acid profiles and chain lengths, but different unsaturation degrees. Although higher degree of unsaturated lipid (C18:1 and C18:2) digests more effectively due to elevated cholecystokinin (CCK) response during *in vivo* digestion. However, the degree of unsaturation appears to be an insignificant factor on *in vitro* lipolysis [323]. The colloidal difference may be the key factor that caused the greater phospholipid digestion. Further, the larger surface area and cation-phospholipid association contribute to the quick access of the lipase to the substrate [327].

Previously, bovine milk phospholipids have been refined for infant formulas to enhance cognitive benefits [126]. The intestinal secretion of pancreatic triacylglycerol lipase (PTL, EC 3.1.1.3), pancreatic phospholipase A_2 (EC 3.1.1.4), and newborns produce much less bile salt

than adults. Instead PTL-related protein 2 and bile salt-stimulated lipase (BSSL, also present in breast milk) are the key lipases responsible for digesting both phospholipids and triacyglycerols in babies' milk in conjunction with gastric digestion [328].

Gas chromatographic analysis of the free fatty acids after milk lipid hydrolysis revealed that oleic acid (C18:1 C9, 22.0 ± 4.0%) was the most abundant fatty acid, followed by myristic acid (C14:0, 19.2 ± 2%), stearic acid (C18:0, 12.5 ± 1.7%), lauric acid (C12:0, 7.3 ± 0.8%), and palmitic acid (C16:0, 4.7 ± 0.8%), which was similar to previous report [38].

Cleavage of phospholipids to release choline

Choline phospholipid breakdown by phospholipases D and A_2, and pancreatic lipases is illustrated in Figure 5-3b at a constant concentration of substrates and enzymes. Phospholipase D (PLD, EC 3.1.4.4) cleaves the choline polar heads of the phosphoric di-ester bonds at the *sn*-3 positions, and can also catalyse transphosphatidylation reactions [329]). Phospholipase A_2 present in pancreatic juice specifically attacks the *sn*-2 acyl position [96]. PLD is an intracellular enzyme, absent in pancreatic juice. Hence phospholipase A_2 and pancreatic lipase had no cleavage effects on choline groups of phospholipids in the present study.

In addition to choline-cleaving phospholipase D, sphingomyelinase (alk-SMase, EC 3.1.4.12) acts on the phosphoric di-ester bond of sphingomyelin, generating ceramide and phosphocholine [99]. Ceramide will be further disintegrated by mucosal ceramidase (N-CDase EC 3.5.1.23) [100].

Choline phospholipid digestion with PLD

Choline released from milk phospholipids exhibited first-order reaction kinetics under the catalysis of PLD, as illustrated in Figure 5-3a. The reaction rate was proportional to the first power of substrate concentration. As the enzyme concentration changed, the reaction rate constants also changed in a range of 0.031 – 0.001 min^{-1}, leading to half-digestion time ranging from 22 – 495 min, as recorded in Table 5-2. These first-order reaction kinetics have been documented in reports on lipolysis of carotenoid-enriched lipids [289] and on the lipolysis of nano-emulsion [330]. PLD plays critical roles in a wide varities of physiological pathways, for instance, membrane trafficking, cytoskeletal reorganisation, regulation of cell proliferation and transformation, and a biomarker of neurodiseases (*i.e.* Parkinson's and Alzheimer's) [331]. Two isoforms PLD_1 (120 kDa) and PLD_2 (106 kDa) have been

characterized, and the latter can be derived from plants (*e.g.* cabbage and carrots), animals (*e.g.* brown spider and venom), and bacteria (*e.g. Ochrobactrum anthropi*) [329].

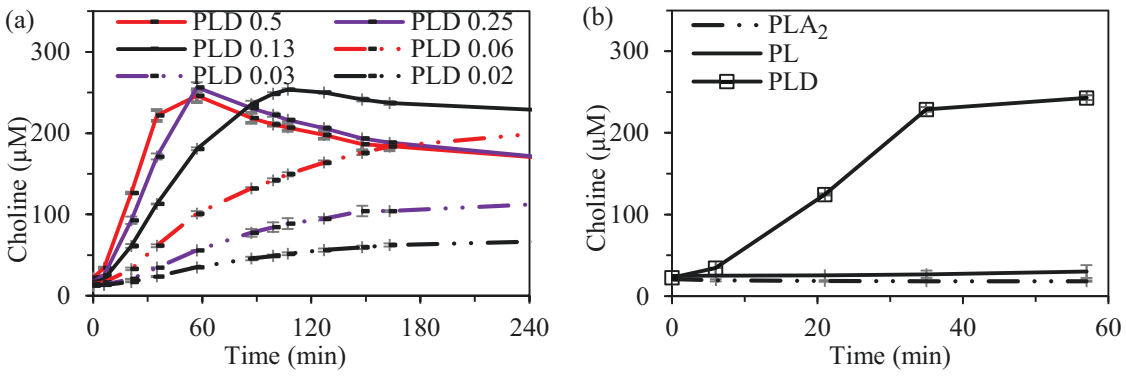


Figure 5-3: Choline cleavage of MPL by three kinds of lipases.

Notes: a: by phospholipase D (PLD); b: by PLD and phospholipase A_2 (PLA_2), and PL; milk phospholipids (MPL); MPL: 1 mg/mL.

Table 5-2: Choline cleavage reaction rate constant of PLD on MPL.

PLD concentration (mg/mL)	Reaction rate constant k ($\times 10^{-2}$ min^{-1})	R^2	Half-digestion time $T_{1/2}$ (min)
0.50	2.7 ± 0.1	0.96	25.6 ± 0.58
0.25	3.2 ± 0.0	0.94	21.5 ± 0.00
0.13	1.6 ± 0.0	0.97	43.2 ± 1.13
0.06	0.6 ± 0.0	0.96	120.9 ± 1.21
0.03	0.3 ± 0.0	0.96	266.5 ± 0.00
0.02	0.13 ± 0.1	0.96	533.1 ± 0.00

Notes: Milk phospholipids (MPL), 1 mg/mL.

5.3.4 Cellular uptake of phospholipids on HT-29 cells

As illustrated in Figure 5-4f, the cellular uptake of milk phospholipids on HT-29 cells was 6.1 ± 0.7%, in line with previous reports on direct diffusion across cell line. For instance, lipolysates (fatty acids and lysophospholipids) are absorbed across epithelial cells, with an absorption rate of more than 90%, and approximately 20% can actively diffuse the mucosa brush border [12]. Also, liposomes are biocompatible, and are able to penetrate into epithelial cells [39]. Most phospholipids are not absorbed intact, and must be digested to permeate the epithelial cells [99]. Additionally, the uptake of fatty acids (hydrolysates of triacylglycerols and phospholipids) is mediated by very low density lipoproteins [332]. Cell membranes are permeable for small molecules, and lipid micelles are too large to cross the cell membrane actively by diffusion [333].

5.3.5 Milk phospholipid antioxidant activity

As illustrated by the DPPH assay (a), hydroxyl radical assay (b) and ABTS assay (c) in Figure 5-4, the antioxidant activity of milk phospholipids was concentration-dependent, in line with previous reports on oil oxidation inhabitation by phospholipids. For instance, phospholipids of egg yolk PC and ox brain PE protected *n*-3 polyunsaturated fatty acids (PUFAs) of salmon oil from peroxidation during storage [334]. Further, 0.5% phospholipid supplementation was found to remarkably alleviate the concentration of volatile organic compounds generated from both salmon and menhaden oils [335]. The primary substrates of free radical reactions include the PUFA moieties [336], amine groups (of PC, SM, PE, or PS) and hydroxyl groups (of phosphatidylglycerol (PG) or PI) in the *sn*-3 side chain of phospholipids [337]. Also, the moieties of hydroxyl and nitrogen-containing amine groups collectively act as catalysts of hydroperoxide decomposition [338], thereby PC, PE, and SM exhibit more antioxidant activity than PS, PG and PI, while phosphatidic acid (PA) was shown to have no antioxidant activity [336]. TBARS results Figure 5-4d further evidenced that the antioxidant activity of milk phospholipids can be used to delay the oxidation of PUFAs.

The CAA assay was developed recently to determine the attenuation effects of antioxidants on the cellular oxidation. As illustrated in Figure 5-4e, the CAA value of milk phospholipids was very low, indicating a poor bioavailability of the antioxidants. The intracellular DCFA was oxidized to fluorescent DCF, which can be reduced with intracellular antioxidants [339].

Milk phospholipids are increasingly regarded as important nutritional ingredients for infant milk formulas to boost cognitive performance of infants [56] as claimed by manufactures, which have been evidenced by either by *ex vivo* model (suckling rats pups [128] and neonatal piglet [115] or by *in vivo* models low birth weight infants [129], infants [130], toddler [116]. In this study, they showed a degree of in vitro antioxidant activity which, in addition to its nutritional benefits, may also be valuable for milk powder preservation (reducing lipid oxidation) during shelf life.

5.3.6 Anti-proliferation activity

Figure 5-4g illustrates that milk phospholipids had no significant effect on HT-29 cell viability. This demonstrates their biocompatibility to HT-29 cells, and is consistent with previous reports on soy lecithin and bovine brain PS. For instance, oral administration of PS exhibited no adverse effects on models of both *in vivo* [314] and *ex vivo* (animal model)

[340]. Also, long-term feeding studies of soy lecithin yielded no treatment-related histologic changes in mice [313] and rats [341].

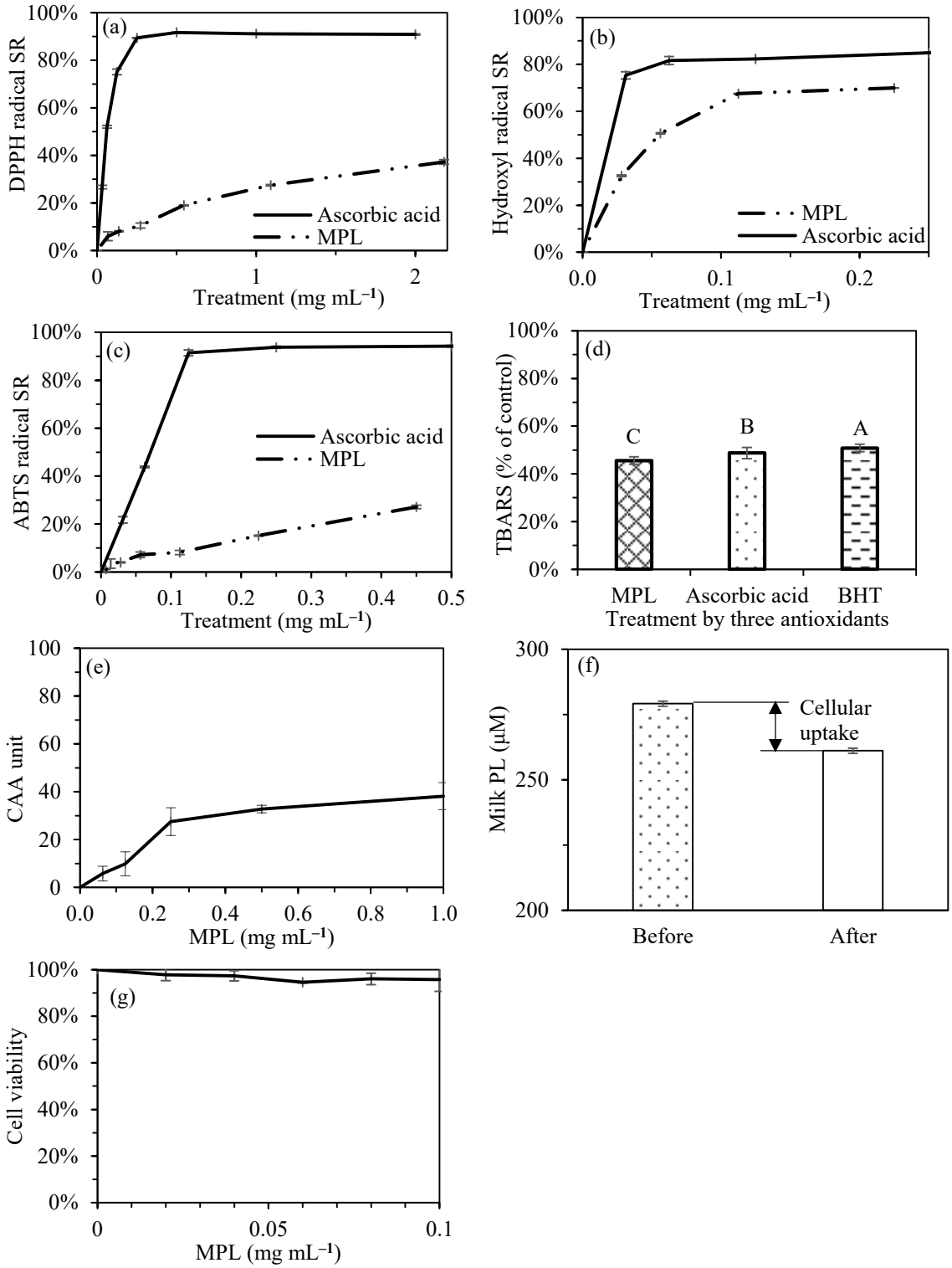


Figure 5-4: MPL antioxidant activity and MTT assay.

Notes: by DPPH radical scavenging (a), hydroxyl radical scavenging (b), ABTS radical scavenging (c), TBARS formation inhibition (d), Cellular antioxidant activity (CAA) assays

(e), milk phospholipid (MPL) concentration before and after cellular uptake (f), and relative cell viability to untreated HT-29 cells by MTT assay (g).

5.4 Conclusion

In the end, milk phospholipid lipolysis followed by first-order reaction kinetics, which were significantly higher ($p < 0.05$) than those of triacylglycerols by pancreatic lipase and fungal lipase. Significant antioxidant activity was evidenced by DPPH, ABTS, TBARS and hydroxyl radical scavenging assays, but their cellular antioxidant activity was very limited. Additionally, their choline groups were not cleaved by pancreatic lipase and they were not absorbed intact during intestinal digestion. This study elaborates on the polarity effects on lipid digestibility and provides useful knowledge on the design of milk phospholipid-fortified functional foods including infant formulas.

Chapter 6: The effects of fungal lipase-treated milk lipids on bread making

Chapter 6 is published as:

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Abstract: Milk lipids have been functional ingredients in bread making. However, incorporation of enzymatically-treated milk lipids into bread recipes and their effects on bread properties has not been explored. This study aims to ascertain the impact of lipase-treated milk lipids on bread quality in comparison with untreated bread controls. As a result of lipase treatment, dough structure was strengthened, and loaf volume improved. *In vitro* digestion revealed that the glycaemic load of lipase-treated breads was significantly lower than that of control samples ($p < 0.05$). In addition, lipase treatment reduced the crumb water activity. The study provides a novel and practical method to improve bread functionalities and lower product glycaemic response, without detrimental effects on the quality of the final products.

Keywords: Rheological characteristics; Texture profile analysis (TPA); Glycaemic response; Complexing index; Anti-staling.

6.1 Introduction

Bread lipids originate from endogenous (i.e. wheat flour) and exogenous (*e.g.* shortening and surfactants) sources. Although they are a minor component, approximately 2 g in 100 g flour [20], lipids play a critical part in bread making. For instance, they have been shown to reduce the crumb firmness [226] and starch digestibility [342] by their interaction with the gluten network and their conjugation with amylose. Filamentous fungal lipases have been isolated and extracted from *Aspergillus* spp. and Rhizopus oryzae for bakery applications [343]. In 2005, Danisco issued a patent to hydrolyse flour lipids with fungal lipase, improving both loaf volume and softness [344]. Lipase treatment reduces fermentation time by virtue of the surfactant effects of lipolysates (mono- and diacylglycerols E471), [345], renders synthetic emulsifiers unnecessary [346], increases oven rise [347], attenuates staling process [226], extends dough durability [348], and promotes gas cell stabilization [227]. Further, lipase treatment of endogenous flour resulted in similar or better dough rheological properties and

crumb texture, by ameliorating the interaction of amylose and lipids compared with diacetyl tartaric acid esters of mono- and diglycerides (DATEM, E472e) and dough constituents, as reported by Colakoglu et al. [346].

Enzyme mixtures can generate hybrid effects on bread quality. For example, the addition of both amylase and lipase to bread mixtures gives rise to appreciable improvement in crumb texture and loaf volume, and significant inhibition of the bread firming process [273]. Combining exogenous lipase implementation (25 mg per kg flour) with supplementary lipid (1 g monoacylglycerols per 100 g flour) led to a higher score in sensory evaluation than untreated samples [349].

Milk lipids are composed of mainly TAG (ca. 98%, only 1% polar lipids), while polar lipids account for ca. half of the total lipid contents, with 22 – 27% galactolipids and 13 – 23% phospholipids [20], which hydrolyse into fatty acids and lyso-phospholipids. Despite of the compositional difference, both lipids have been used for baked goods.

In respect of roles of milk fat-derived bakery lipid, first, milk lipid hydrolysates (i.e. MAG, fatty acids) have been proven to be dough strengtheners and bread textural improvers, via such mechanisms as starch-lipid complexes [174], emulsification effects of MAGs [350], and shortening effects of un-hydrolysed TAG [23]. Whereas, flour lipid hydrolysates (*e.g.* lyso-phospholipids) can be surfactants of bread dough [190]). Therefore, milk fat performs more functionalities than flour lipids in bread making. Second, flour lipid alone didn't improve loaf volume. Using various combination of polar lipids and neutral lipids on defatted flour, Schaffarczyk et al. [351] found that the hydrolysate of polar lipids of baking lipases needed the presence of unmodified, non-polar lipids (as shortening fat) to provide optimal functional effects. Further, it has been found that the major phospholipid subclass—phosphatidylcholine only exhibited a neglect impact on baking activity of wheat flour [352]. Third, compared to ca. 1 g flour lipids per 100 g flour, milk fat has more abundance in bread recipes (*ca.* 4.15 g per 100 g flour for AACC10.10 recipe).

Given the above three reasons plus the industrial availability and nutritional value of milk fat [353], hence, it can be hypothesized that lipase-treated milk lipids may perform greater function than flour lipids. Thus far, using exogenous lipase-treated milk fat as bread improvers has not yet been explored. Therefore, in the present investigated by evaluating dough rheological characteristics and pH, bread physical and textural properties, *in vitro* digestion and predictive glycaemic response, starch-lipid complexing index (CI), and anti-

staling performance. The starch-lipid interaction induced by lipase-treatment to milk fat was elaborated as well.

6.2 Materials and methods

The materials and methods in the section of 6.2 can be referred back to the corresponding sections in Chapter 3, as illustrated in Table 6-1.

Table 6-1: Materials and methods for lipase-treated milk lipid in bread recipes.

Section 6.2	Materials and methods	As mentioned in the section of
6.2.1.	Dough preparation and bread making	3.13
6.2.1.1.	Ingredients and reagents	3.13.1
6.2.1.2.	Bread recipe	3.13.2
6.2.2.	Dough characteristics	3.14
6.2.2.1.	Dough rheological properties	3.14.1
6.2.2.2.	Dough stickiness	3.14.2
6.2.2.3.	Dough firmness	3.14.3
6.2.2.4.	Dough pH	3.14.4
6.2.3.	Bread analysis	3.15
6.2.3.1.	Physical properties	3.15.1
6.2.3.2.	Crust colour	3.15.2
6.2.3.3.	Textural profile analysis (TPA)	3.15.3
6.2.4.	Bread *in vitro* digestion and glycaemic glucose equivalent (GGE)	3.16
6.2.5.	Complex index (CI)	3.17
6.2.6.	Anti-staling performance	3.18
6.2.6.1.	Water activity and bread firming during shelf life	3.18.1
6.2.7.	Lipolysis and free fatty acid (FFA) determination	3.19
6.2.8.	Statistical analysis	3.37

6.3 Results and discussion

6.3.1 Lipolysis

Lipopan F, a second generation baking phospholipase (E.C. 3.1.1.X) extracted from Fusarium oxysporum strain by Novozymes, has lipolytic activity on both triacylglycerols [354] and phospholipids as reported by Gerits et al. [347]. It cleaves two fatty acids from each substrate molecule (i.e. both triacylglycerols and phospholipids) [288, 355]. In respect to endogenous flour lipids, the dose of Lipopan F has been optimized by Moayedallaie et al. [345] as 15 mg/kg flour (563 U/kg). The degree of milk fat hydrolysis in 20 min was 7.60 ± 0.82%, 12.11 ± 1.89%, and 19 ± 2.06% for LML4.5, LML9, and LML18, respectively. The generated fatty acids were 0.10 g, 0.16 g, 0.26 g per 100 g dough (acceptable at level of less than 0.3 g per

100 g dough). The produced mono- and diacylglycerol (0.14 – 0.34 g per 100 g flour) fell into the surfactant supplementation level of 0.3 – 1.0 g per 100 g flour, as depicted for bread recipes by Pareyt et al. [20].

Both the reaction rate constant k and hydrolysis degree of milk fat increased with the increase of lipase dose, as illustrated in Table 6-1 and Figure 6-1. When the lipase dose increased, the reaction rate constants increased accordingly, leading to the reduction of half lipolysis time ($T_{1/2}$). Both polar lipids and non-polar lipids hydrolysis can be catalysed by Lipopan F. The half lipolysis time of LML9 samples was 253.43 ± 32.37 min in Table 6-1. According to the measured reaction rate constants, the amount of fatty acid produced can be modulated by changing the level of lipases and substrates (i.e. milk fat) or the time of lipolysis.

Lipase treatment on flour lipids (1.2 g per 100 g flour), which is usually implemented in baking factories, appears to be easier to the treatment of milk fat. However, milk fat (4.15 g lipids per 100 g flour) may exhibit more potentials, and therefore, a side control (LML2.3) was designed to depict the effectiveness of lipase-treatment. The present lipase-treated milk lipids are not suitable for long fermentation baking due to free fatty acid accumulation, but they are suitable for the replacement of synthetic emulsifiers in industrialized bakery manufacturing, in which lipase treatment time can be controlled precisely.

Table 6-2: Milk lipid lipolysis reaction rate constants and half digestion time.

Samples	k **(min^{-1})**	$T_{1/2}$ **(min)**	**R^2**
LML4.5	0.002^{c} ± 0.001	289.80^{a} ± 21.82	0.96
LML9	0.003^{b} ± 0	253.43^{b} ± 32.37	0.95
LML18	0.004^{a} ± 0.001	184.39^{c} ± 40.37	0.96

Notes: The results are presented as means ± standard deviations ($n = 3$). Means in the same row that do not share a letter are significantly different ($p < 0.05$). Lipase dosage for LML4.5/9/18 were 4.5, 9 and 18 mg for 7.5 g of milk butter, respectively. k is reaction rate constant (min^{-1}) and $T_{1/2}$ is half digestion time (min).

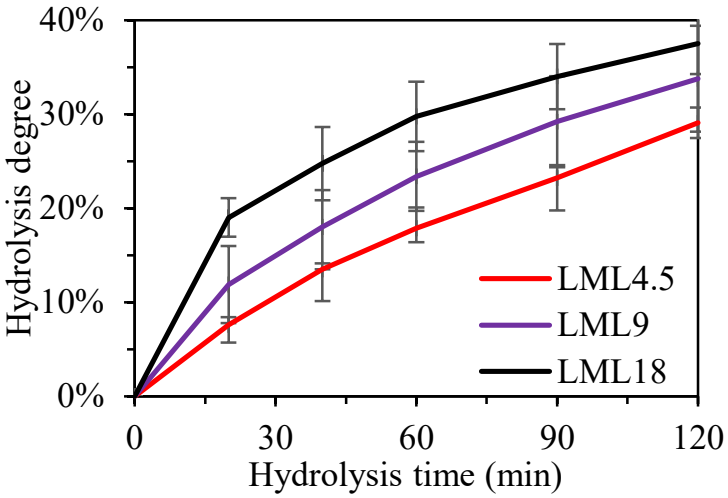

Figure 6-1: Lipolysis of milk lipids.

Notes: $n = 3$; Lipase dose for LML4.5, LML9 and LML18 were 4.5, 9, and 18 mg for 7.5 g milk butter.

6.3.2 Dough characteristics

Dough resistance to extension and extensibility

As illustrated in Table 6-3, dough rheological properties were altered as a result of lipase treatment. The proportion of resistance strength to extensibility (R/E) significantly ($p < 0.05$) increased in LML4.5 samples, while R/E was reduced slightly in other samples, compared to controls. This is consistent with a previous report on surfactant-modified dough, where the maximum R/E was 2.01 at a 0.3 g addition of monoacylglycerols to 100 g flour. However, overdose 0.4 g addition of monoacylglycerols to 100 g flour led to a weaker dough [356]. This is also in support of another report on DATEM-emulsified bread dough [357], where this ratio was increased by 28% for an addition of 0.05 g DATEM to 100 g flour, but if the DATEM concentration increased to 0.1%, the R/E decreased to that of the control.

In regards to the strengthening effect of using surfactant, it has been found that, with the addition of surfactant, the dough rheological properties also increased in a recent report [358]. In a more recent study on lipase impact on dough rheological, a direct mechanism for dough strengthening was attributed to improved surface activity at gas-liquid interface and lipid surfactant stabilization of the liquid lamellae surrounding gas cells [351]. The film generated remarkable substantial elastic restoring forces, which resist distortion at the interfacial area of gas-liquid. In another emulsifier study, both distilled monostearate and lecithin stabilized the shortening crystals and increased the air-absorbing ability on both beef tallow and hydrogenated palm oil by gas absorbing capacity measurement [180]. Also, surfactant-gluten

hydrophobic interactions led to protein aggregates and strengthened the gluten network [356]. During the dough mixing, starch particles hydrate and swell, and gluten develops into continuous extensible gluten film by hydration and lipid coating, the lipid crystals decrease the surface tension of the gluten film (lubrication effects), promoting aeration of the dough [23].

Dough firmness

The dough firmness decreased in all the lipase-treated samples by 7 – 25% (Table 6-3), consistent with a report by Mehta *et al*. [359], in which 2, 4, and 8% of lipid fortification led to a reduction of the dough density by 0.06 – 0.07 g/mL from 1.22 g/mL. Whereas the density of dough reduced by more than 5%, the dough texture became looser and less firm after lipid treatment. In addition, surfactants (distilled mono-acylglycerol i.e. monostearate and lecithin) stabilized the bakery lipid crystals and increased the index of air-absorbing ability on shortening oil (hydrogenated palm oil) by various degree [180]. It has been well known that, during dough mixing, the gluten network develops and the nucleation of gas bubbles occurs concomitantly. Dough will be softer as a result of elevated gas bubble entrainment capacity by surfactants, as also elaborated at the above section (Dough resistance to extension and extensibility).

Dough stickiness

Dough stickiness, a parameter associated with the dough machinability, was reduced in LML samples (Table 6-3), in agreement with a report by Colakoglu *et al*. [346], in which dough stickiness decreased by approximately 13% when Lipopan F lipase (dose: 10 mg per kg flour) was added to treat whole flour, while the increase of dose to 20 – 40 mg/kg (flour base) did not further reduce the dough stickiness. This was in accordance with a previous report in which soy lecithin supplementation of 0.2 – 0.5% reduced dough stickiness significantly [360].

Dough pH

The acidity levels of lipase-treated doughs were 0.3 – 0.9 lower than those of controls due to yielded fatty acids, but still falling in the range of pH 4.5 – 5.5, *i.e.* a normal range of yeasted dough [361], thus baking yeast activity was not affected. Further accumulation of free fatty acids can cause an off-flavour, as recorded by Brunstedt *et al.* [362]. Hence, the amount of free fatty acids from lipolysis should be limited by modulating the dose of lipases and milk lipids or treatment time.

Table 6-3: Dough textural characteristics and dough pH.

Samples	Control	LFL2.3	LML4.5	LML9	LML18
Resistance to extension (g)	$45.43^{b} \pm 0.96$	$29.85^{d} \pm 0.98$	$70.28^{a} \pm 0.71$	$46.18^{b} \pm 0.39$	$41.74^{c} \pm 0.29$
Extensibility (mm)	$54.83^{b} \pm 0.19$	$56.72^{b} \pm 2.16$	$23.92^{c} \pm 0.71$	$57.07^{b} \pm 1.10$	$74.89^{a} \pm 0.04$
R/E (g/mm)	$0.83^{b} \pm 0.02$	$0.53^{c} \pm 0.02$	$2.94^{a} \pm 0.09$	$0.81^{b} \pm 0.01$	$0.56^{c} \pm 0.00$
Firmness (g)	$301.04^{a} \pm 0.83$	$207.92^{c} \pm 7.81$	$287.68^{ab} \pm 4.47$	$278.64^{b} \pm 7.91$	$274.45^{b} \pm 3.92$
Stickiness	$32.38^{a} \pm 0.22$	$25.37^{b} \pm 0.30$	$20.35^{d} \pm 0.95$	$19.47^{d} \pm 0.69$	$22.08^{c} \pm 0.05$
pH	$6.08^{a} \pm 0.07$	$5.81^{a} \pm 0.01$	$5.95^{a} \pm 0.30$	$5.85^{a} \pm 0.02$	$5.23^{b} \pm 0.08$

Notes: The results are presented as means ± standard deviations ($n = 3$). Means in the same row that do not share a letter are significantly different ($p < 0.05$). Lipase dosage for LML4.5/9/18 were 4.5, 9 and 18 mg for 7.5 g of milk butter, respectively. Lipase dosage for LFL2.3 were 2.3 mg for 150 g wheat flour. R/E: ratio of resistance strength to extensibility.

6.3.3 Bread analysis

Bread physical properties

In addition to dough-strengthening resulting from lipase-treatment, loaf volume increased due to the effects of gas cell stabilisation by milk lipid emulsifiers. As shown in Table 6-3, the enzymatic hydrolysis led to a 5 – 11% increase in bread volume except for LML18. Crumb porosity also increased in LFL 2.3 and LML 9 samples except for LML 18, which was in agreement with a study on both lipase-treated breads by Gerits *et al*. [190] and the breads from food surfactant-modified dough (sucrose ester E473, [363]). Substitution with 4 g palm oil per 100 g flour as shortening reduced the crumb density by 4%, by improvement of porosity, as reported by Chin *et al*. [166]. In addition, the rise of loaf volume was attributed to the improvement of both diameter by 10% and homogeneity of pores by at least 5%, as reported by Søe *et al.* [344]. The specific volume for the controls and lipase-treated samples LML9 were 3.73 ± 0.05 mL/g and 4.10 ± 0.06 mL/g, respectively, consistent with a report by Gerits *et al*. [227], where the loaf volume increased by *ca.* 30.8% and 21.7 ± 4.3% for lipase-treatment of flour lipid (1 mg lipase protein/kg flour) and DATEM addition (0.5 g in 100 g flour), respectively. It was also in support of a lipase treatment study by Moayedallaie *et al*. [345], where loaf volumes were 4.0 ± 0.19 mL/g and 4.5 ± 0.18 mL/g for control and treatment, respectively. Further increase in lipase dose or treatment time (*e.g*. long fermentation) didn't increase loaf volume (*i.e*. LML18), as evidenced in previous reports by Moayedallaie et al. [345] and Gerits et al. [227]. However, overdose (LML18) reduced loaf size, similar to a lipase-treated bread report, where loaf volume was optimized at 1 mg

Lipopan F per kg flour, where 5 mg/kg dose significantly decreased the volume to 53.53 cm^3 from the maximum (57.40 cm^3) [190], probably due to the loss of gas cell stability and subsequent coalescence or collapse when reaching the limitation of porosity at high concentrations.

Crust colour

As shown in Table 6-4, the brownness indexes (BI) of lipase-treated breads were remarkably higher than those of controls, and the WI values significantly decreased in contrast to controls ($p < 0.05$). Also, similar colour results were observed in a report on 0.1 – 0.3 g surfactant-supplementation (based on 100 g flour) to cassava-maize-wheat bread by Eduardo *et al*. [271], in which the BI value significantly increased ($p < 0.05$) from 80 to 88.8 – 87.9 and 84.1 – 84.8 for DATEM and sodium stearoyl lactate (SSL, E481), respectively. Also, both DATEM and lipase treatment produced darker bread than the control [345]. In addition, in a study on surfactant treatment on bread quality, the crust colour was dependent on both the gluten content and the type of surfactants (DATEM, SSL, and monoacylglycerols) as reported by Chin *et al*. [364]. BI values are related to both the Maillard reaction and starch dextrinization/sugar caramelisation [365]. The Maillard reaction is determined by temperature, reaction time, pH, composition, and water activity (optimum at 0.65 – 0.75), and an increase of water activity to a certain level can increase the rate of Maillard reaction as well. However, the reaction rate will decrease when water activity increases further [366]. It is likely that lipase treatment in the LML samples lowered the water binding capacity of starch, thus enhanced the Maillard reaction and generated darker bread.

Complexing index

As illustrated in Table 6-4, the complexing index (CI) value was dose-dependent, and the value for LML9 was lower than that of LML18 and LFL2.3, higher than that of LM4.5, and comparable with a previous study by Lau et al. [342]. It has been well known that amylose-lipid complexes reduce iodine-binding capacity of starch since fatty acids and monoacylglycerols compete with iodine to fill the helix cavities of starch molecules, while triacylglycerols form no complex with starch [367]. The total starch of bread samples were 42.9 ± 1.2%, in agreement with the results of controls by Liu et al. [267].

Texture profile analysis

As illustrated in Table 6-4, the firmness and chewiness of bread crumb for treated samples were significantly reduced, compared to the control samples, hence bread became softer through modification of milk fat. The controls had the firmest texture, while LML9 and

LML18 samples were softer than other samples. Further, the softening effect in LML4.5 and LFL2.3 was less pronounced, in contrast to LML9 and LML18. The TPA results were also consistent with a previous report on lipase- or surfactant-treated breads by Gerits et al. [226], in which both Lipopan F and SSL led to improvement in crumb tenderness. In addition to firmness, crumb chewiness was impacted more than the other TPA parameters, in particular, springiness, cohesiveness and resilience in all lipase-treated samples. The chewiness of controls was higher than other samples, with a similar profile to the firmness ($r = 0.99$), consistent with a report on the positive correlation of crumb chewiness and firmness ($r = 0.96$) [273]. Also, the crumb resilience was found to be highly correlated to the firmness ($r = 0.90$). The TPA results were in agreement with a previous report on bread TPA, where larger loaves were softer and had a looser loaf structure (i.e. higher porosity), resulting in lower firmness and chewiness values as recorded by Liu et al. [267]. Apart from the gas cell stabilisation, the composition of free fatty acids were highly correlated ($r = 0.98$ and 0.91 for FFA in the free and the bound lipid fraction, respectively) with the loaf volume [190]. Surfactants like mono- diacylglycerols can form starch-lipid complexes with amylose during baking, and it may also have a softening effect on bread crumb [20, 226]. Rogers et al. [368] observed that MAG can conjugate with amylose and TAG cannot. As textural improvers, MAG and TAG had different mechanism. MAG can form starch-lipid complexes to soften texture of crumb, while TAG tenderizes crumb texture by confinement of gluten protein network development [20].

During baking, starch particles become gelatinized and the gluten film turns into a permanent cross-linked thin film, together with shortening lipids [369]. Monoacylglycerols are typical crumb softeners via an inclusion complex mechanism. Amylose-monoacylglycerol inclusion complexes are water-insoluble, therefore the complexed amylose does not participate in gelatinisation. Thus upon cooling after baking, the complexed amylopectin will not recrystallize, and it will not contribute to the staling of the bread crumb [26]. Also, amylose-lipid complexes have been attributed to limit starch swelling and amylose crystallization (i.e. gelation) and to thereby impede crumb firmness [226].

Table 6-4: Bread physical properties, digestibility, and firming rate during storage.

Samples	Control	LFL2.3	LML4.5	LML9	LML18
Volume (mL)	$167.83^{c} \pm 1.61$	$177.50^{b} \pm 0.50$	$171.33^{c} \pm 1.53$	$183.67^{a} \pm 1.53$	$162.33^{d} \pm 2.08$
Density (g/mL)	$0.27^{ab} \pm 0.00$	$0.25^{c} \pm 0.00$	$0.26^{b} \pm 0.00$	$0.24^{d} \pm 0.00$	$0.27^{a} \pm 0.00$
SV (mL/g)	$3.73^{cd} \pm 0.05$	$3.97^{b} \pm 0.02$	$3.81^{c} \pm 0.04$	$4.10^{a} \pm 0.06$	$3.65^{d} \pm 0.04$
Porosity	$0.76^{cd} \pm 0.00$	$0.77^{b} \pm 0.00$	$0.76^{c} \pm 0.00$	$0.78^{a} \pm 0.00$	$0.75^{d} \pm 0.00$

Height (mm)	$53.91^{ab} \pm 0.81$	$56.50^{a} \pm 0.65$	$52.83^{bc} \pm 1.24$	$56.24^{a} \pm 1.18$	$50.32^{c} \pm 0.90$
L^*	$73.64^{a} \pm 2.19$	$70.97^{a} \pm 0.62$	$62.30^{b} \pm 0.29$	$65.42^{b} \pm 0.40$	$64.44^{b} \pm 2.18$
a^*	$2.80^{b} \pm 0.75$	$4.79^{b} \pm 0.28$	$9.22^{a} \pm 1.78$	$8.26^{a} \pm 0.79$	$7.93^{a} \pm 0.60$
b^*	$24.67^{b} \pm 0.90$	$26.32^{ab} \pm 0.26$	$28.12^{a} \pm 1.86$	$28.71^{a} \pm 0.84$	$27.33^{ab} \pm 1.05$
WI	$63.77^{a} \pm 2.20$	$60.51^{a} \pm 0.37$	$52.04^{b} \pm 1.23$	$54.29^{b} \pm 0.42$	$54.42^{b} \pm 1.23$
BI	42.75 ± 4.05^{b}	50.27 ± 0.56^{b}	69.47 ± 6.89^{a}	65.65 ± 2.61^{a}	62.98 ± 1.13^{a}
Firmness (g)	$337.43^{a} \pm 3.38$	$251.78^{b} \pm 4.99$	$196.41^{cd} \pm 7.28$	$187.10^{d} \pm 2.00$	$206.35^{c} \pm 8.89$
Springiness	$0.90^{ab} \pm 0.04$	$0.83^{bc} \pm 0.05$	$0.93^{a} \pm 0.02$	$0.79^{c} \pm 0.02$	$0.92^{a} \pm 0.02$
Cohesiveness	$0.62^{a} \pm 0.01$	$0.57^{ab} \pm 0.02$	$0.56^{ab} \pm 0.02$	$0.60^{ab} \pm 0.02$	$0.54^{b} \pm 0.04$
Chewiness (g)	$187.51^{a} \pm 7.61$	$118.51^{b} \pm 11.86$	$102.59^{b} \pm 5.33$	$89.27^{bc} \pm 1.04$	$103.07^{bc} \pm 4.72$
Resilience	$0.28^{a} \pm 0.01$	$0.22^{b} \pm 0.03$	$0.23^{ab} \pm 0.02$	$0.24^{ab} \pm 0.01$	$0.21^{b} \pm 0.01$
CI		$0.17^{ab} \pm 0.01$	$0.05^{c} \pm 0.01$	$0.11^{b} \pm 0.03$	$0.19^{a} \pm 0.02$
WA	$0.96^{a} \pm 0.005$	$0.96^{a} \pm 0.001$	$0.96^{a} \pm 0.004$	$0.94^{b} \pm 0.004$	$0.92^{c} \pm 0.003$
IFDF (g/day)	$67.93^{a} \pm 2.04$	$29.74^{c} \pm 0.89$	$28.74^{c} \pm 0.86$	$14.49^{d} \pm 0.43$	$36.48^{b} \pm 1.09$
AUC	$457.20^{a} \pm 5.73$	$406.42^{c} \pm 15.75$	$423.48^{b} \pm 5.31$	$422.85^{b} \pm 5.84$	$385.68^{d} \pm 14.75$

Notes: Means ± standard deviations ($n = 3$). Means in the same row that do not share a letter are significantly different ($p < 0.05$). LFL2.3 was referred to samples with flour (150 g) lipid treatment by 2.3 mg lipase; lipase dosage for LML4.5/9/18 represented 4.5, 9 and 18 mg for 7.5 g of milk butter, respectively. LFL2.3: 2.3 mg lipase for 150 g wheat flour; SV: specific volume (mL/g); WI: whiteness index; BI: brownness index; CI: complexing index; WA: water activity; IFDS: increased firmness during staling.

6.3.4 *In vitro* digestion and predictive glycaemic response

As illustrated in Figure 6-2, both the area under curve (AUC, for the reducing sugar curve) and reducing sugar concentration of all lipase-treated samples were lower than those of the control ($p < 0.05$). The lipase-treated samples were significantly more resistant to hydrolysis than controls, and the complexing index was highly correlated ($r = 0.94$) to AUC for the reducing sugar curve, in agreement with the report by Lau *et al.* [342], where 20 g addition of coconut, grapeseed, and olive oil to 100 g flour yielded CI values of 30 – 50% in bread, thereby reducing bread glycaemic index significantly ($p < 0.05$). As described previously by Chao *et al*. [174], forming resistant starch using starch-lipid complexes has been a viable tool to lower the glycaemic response of cereal products. Lipase treatment improves both textural and physiological properties of bread, and whereas, α-amylase addition increases both glycaemic index and loaf size [370].

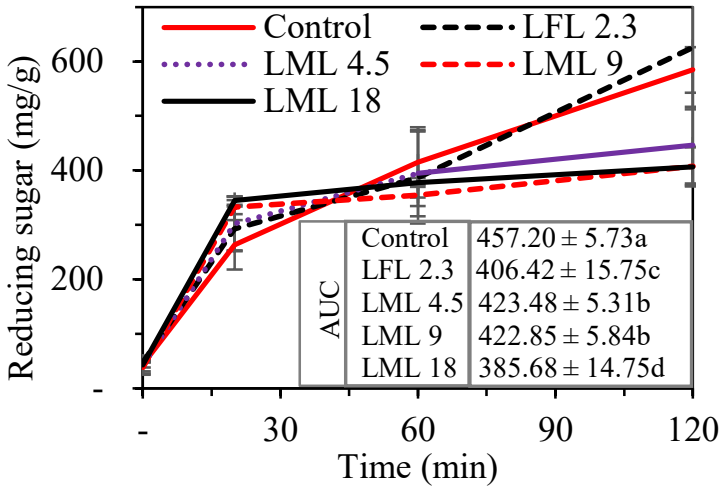

Figure 6-2: Reduced sugar release *in vitro* digestion and AUC.

Notes: Means ± standard deviations ($n = 3$). Means in the same column that do not share a letter are significantly different ($p < 0.05$). LFL2.3 was referred to samples with flour (150 g) lipid treatment by 2.3 mg lipase; lipase dosage for LML4.5, LML9, and LML18 were 4.5, 9 and 18 mg for 7.5 g of milk butter, respectively. AUC: area under the curve of reducing sugar.

6.3.5 Water activity and bread staling

As illustrated in Table 6-4 and Figure 6-3, the water activity (an indicator of bread stability, quality, and firming rate during storage) of LML9 samples was reduced by 4% in contrast to that of the controls, in line with a previous study on enzymatically treated bread [273]. The reduction in water activity is related to lipophilicity change by surfactants [371]. Similarly, as a result of lipase-treatment or addition of surfactant (i.e. sodium stearoyl lactylate (SSL), reduction in crumb water activity was evidenced by a decrease in 1H nuclear magnetic resonance (NMR) population in fresh bread crumb with lipase or SSL treatment [226]. During storage, water migrates from crumb to crust, leading to amylopectin crystallization and stiffness of gluten network [372], and therefore, reducing water migration can delay the rate of crumb hardening.

Along with the reduction of water activity, the staling of lipase-treated samples (recorded as firmness) was significantly reduced ($p < 0.05$) in contrast to the controls (Fig. 3). This is consistent with previous reports on lipase treatment by Gerits et al. [226] and Purhagen et al. [192], and enzyme mixture treatment [373]. Additionally, amylose-lipid complexes can hinder the crystallisation (i.e. gelation) of amylose (i.e. decrease of crystal populations) during the storage of bread, thereby limiting the initial firmness of bread, while amylopectin

retrogradation was impaired by amylose complexation with lipolysis products (*e.g.* free fatty acids, or lyso-lipids) as determined by low-field nuclear magnetic resonance [226].

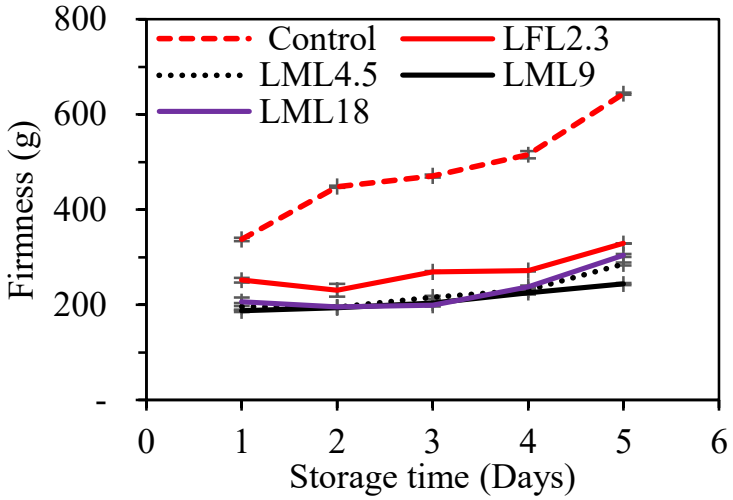

Figure 6-3: Bread crumb firmness during storage.

Notes: LFL2.3 was referred to samples with flour (150 g) lipid treatment by 2.3 mg lipase; lipase dosage for LML4.5, LML9, LML18 were 4.5, 9 and 18 mg for 7.5 g of milk butter, respectively.

6.4 Conclusions

The current study investigated milk lipolysate effects on bread quality, digestibility, and staling. Lipase treatment strengthened the textures of both dough and bread. In addition to oven rise, amylose-lipid complexes reduced the starch digestibility. With the decrease of crumb water activity, the firming rate of bread crumb was reduced. The dough structure greatly impacted on the bread textural properties including porosity. On the whole, this study demonstrates the potential application of exogenous fugal lipase-treated milk lipids to improve bread quality and reduce its glycaemic index.

Chapter 7: Lipase-treated milk lipids on wheat, corn, and rice starch digestibility and functionalities: Amylose-milk fatty acid complex reduces glycaemic index

Chapter 7 is published as:

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Abstract: Fatty acids of plant oils have been used to prepare resistant starch. Only a few papers have been published on how milk lipid-starch complexes affect starch digestibility. This study used fungal lipase-treated milk lipids to prepare starch-fatty acid complexes and the complexes were characterized spectroscopically. An empirical equation was proposed to estimate the complexing index (CI), and spectroscopy analysis verified the conformation of the milk fatty acid-starch complexes. Rheological analysis revealed that the starch-fatty acid complexes had decreased peak viscosities and gel firmness, while increasing the final viscosities significantly ($p < 0.05$). As a result of complexing, the starch digestibility was reduced by 19% for corn starch, 17% for wheat starch, and 25% for rice starch with milk fatty addition (12%, w/w) in *in vitro* studies. The study together with its results provide a means for manufacturing resistant starches using milk lipids.

Keywords: Glycaemic index, amylose-fatty acid complex, *in vitro* digestion, rheological characteristics; enzyme kinetic

7.1 Introduction

Starch from different origins has already been used to prepare conjugations with lipids to prepare resistant carbohydrate foods. For instance, brown lentil-plant fatty acid complexes (10%, w/w) increased the content of resistant starch of brown lentil by 3% – 6.4% [374]. Recently, lotus seed-fatty acids (C8 – C18, 5% w/w) resulted in complexing indexes of 11.8 – 86.3%, corresponding to increases of 12% – 112% in resistant starch content [375]. Also, bambara groundnut starch-fatty acid complexation (4%, w/w) yielded resistant starch with complexing index of 30% – 65% with stearic acid, linoleic acid, and lysophosphatidylcholine

[376]. Furthermore, pea amylose extract may be complexed with fatty acids (10%, w/w) of various chain lengths (C10 – C18) at 30, 50 and 70˚C in potassium hydroxide solution [377]. In addition to pseudocereals, maize starch has been used to conjugate with lipids, yielding CI values of 96.3, 41.8, 8.3, and 1.1% for monopalmitate glycerol, palmitic acid, tripalmitate glycerol, and dipalmitate glycerol, respectively [174]. Further, rice starch-fatty acid complexes (20%, w/w, C4 – C18 fatty acids) were used to produce edible film, yielding CI value of 36.54 ± 2.5% to 15.68 ± 2.2% [378]. Wheat flour and oleic acid formed starch-lipid complexes at 128.3˚C by extrusion [379].

Starch-fatty acid complexes have been used as biodegradable delivery systems for bioactive molecules [377], in the Preparation of edible films for food preservation [378], inhibition of bakery staling [380], and in the preparation of low glycaemic response foods [342].

Middle and long chain fatty acids (*e.g.* palmitic acid, myristic acid, lauric acid, stearic acid, and linoleic acid from plant oils) have sufficient complexing capacity with starches, while tri- and diacylglycerol have virtually no complexing ability with starches. To our knowledge, thus, milk fats have not been utilized for complexing with starch because triacylglycerols do not conjugate with amylose and amylopectin. Milk butter (per 100 g, USDA code 1145) is rich in middle- and long-chain fatty acids (*e.g.* C12:0 2.587 g; C14:0 7.436 g; C16:0 21.697 g; C18:0 9.999 g; C18:1 20.4 g [18]), and it has been widely used in the food industry due to its cost efficiency and availability. Therefore, in the present study, milk fatty acids are produced from milk butter by fungal lipase hydrolysis to conjugate with rice, corn, and wheat starches by cooking, and their complexes were evaluated by CI determination, spectroscopy, rheology technique, and *in vitro* digestibility.

7.2 Materials and methods

The materials and methods in the section of 7.2 can be referred back to the corresponding sections in Chapter 3, as illustrated in Table 7-1.

Table 7-1: Materials and methods for starch-milk lipid complexes.

Section 7.2	**Materials and methods**	**As mentioned in the section of**
7.2.1.	Materials and reagents	3.20
7.2.2.	Preparation of starch-milk fatty acid complexes	3.21
7.2.3.	Light microscopy analysis	3.22
7.2.4.	Rheological properties of starch gels	3.23
7.2.4.1.	Rapid viscosity analysis (RVA)	3.23

7.3 Results and discussion

7.3.1 Milk lipid treatment with fungal lipase

The milk lipids were digested overnight and their hydrolysis reaction rate constants were measured to be 0.007 ± 0.001 min^{-1}, corresponding to a half-digestion time 97.69 ± 8.88 min (R^2 = 0.97). During the overnight lipolysis, the degree of hydrolysis was greater than 99%. The gas chromatographic analysis of free fatty acids after milk lipid hydrolysis revealed that oleic acid (C18:1, 28.56 ± 5.08%) was the most abundant fatty acid, followed by myristic acid (C14:0, 19.18 ± 2.04%), stearic acid (C18:0, 12.50 ± 1.69%), palmitic acid (C16:0, 7.82 ± 1.39%), and lauric acid (C12:0, 7.30 ± 0.81%). Thus the mid-chain fatty acids dominated the profile of fatty acids, consistent with USDA data (#1003) [18].

7.3.2 Light microscopy analysis and particle size distribution

As illustrated in Figure 7-1(f – h), in all three kinds of starch-milk fatty acid complexes (MFA12, 12% milk fatty acids, starch based), most starch particles fully gelatinized during hydration and heating with some particles still gelatinizing, and furthermore, Sudan black-stained milk lipids were distributed densely and evenly in the starch gel matrices, as also observed in reports on corn starch [381] and wheat starch [382]. When starch dispersions were heated to 95˚C, the starch particles swelled irreversibly, and amylose leached into aqueous phase concomitantly. Losing crystalline structures, the starch particles became amorphous, which was reflected by the disappearance of birefringence and Maltese cross in the optical microscopic images of corn (Figure 7-1f) and rice (Figure 7-1g) starch gels. Finally, the starch dispersions turned into viscoelastic gels, in which amylose and water were bound by hydrogen bonding in three-dimension networks. As can be seen in the corn starch gel of Figure 7-1a. Upon cooling and gelation, amylose and amylopectin recrystallized successively [383]. Minor un-gelatinized particles with vague Maltese crosses (3 – 8 μm in diameter for rice starch in Figure 7-1g and approximately 20 μm in diameter for corn starch in

Figure 7-1f, consistent with the average particle size ($d_{3,2}$) of rice starch 5.46 ± 0.02^{e} μm in Table 7-2 suggested that rice starch gelatinized slower than wheat and corn starches, likely due to their weaker swelling power than those of the latter. The average particle sizes ($d_{3,2}$) of high amylose corn starch and waxy corn starch were 12.70 ± 0.36^{d} μm and 17.93 ± 0.06^{b} μm (Table 7-2), respectively, compatible with a recent report [275]. The particle of corn starch is bigger than rice starch, and finer than wheat starch, consistent with the present optical observations in Figure 7-1(a – e).

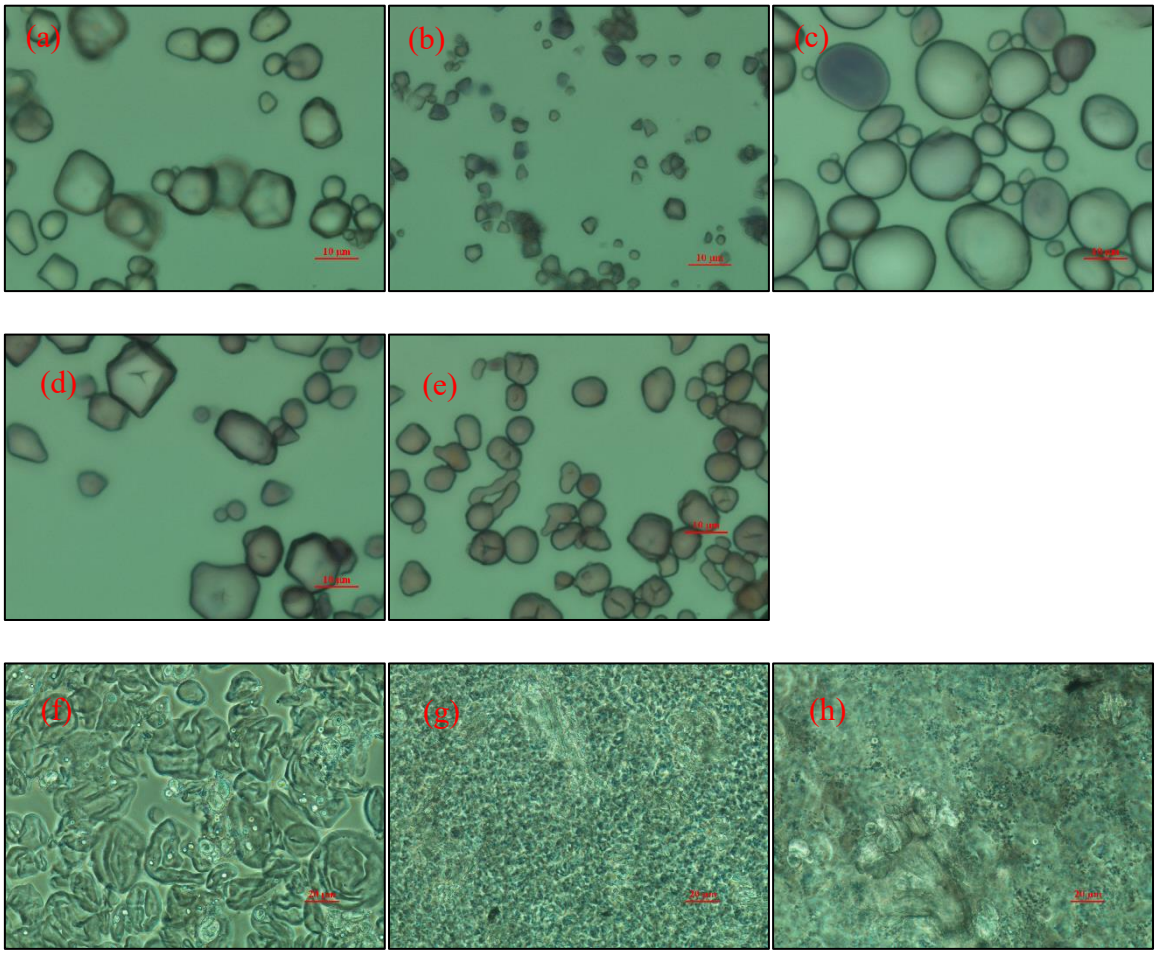


Figure 7-1: Light microscopy of starch-milk fatty acid complexes.

Notes: (a – e) were viewed at 1000-fold magnification, bright field optical microscopy for five kinds of iodine-stained starches, including (a) corn starch, (b) rice starch, (c) wheat starch, (d) waxy corn starch, and (e) high amylose corn starch; (f – h) were viewed at 400-fold magnification, bright field phase contrast II optical microscopy for three kinds of starch-stabilized milk fatty acids (MFA 12%, w/w, starch based, stained by Sudan black dye), such as (e) corn starch, (f) rice starch, and (g) wheat starch.

Table 7-2: The specific surface area and sizes of starch particles.

Starch	Specific Surface Area (m^2/kg)	$d_{3,2}$ (μm)	$d_{4,3}$ (μm)
RS	$1{,}100.67^{a} \pm 3.06$	$5.46^{e} \pm 0.02$	$8.45^{e} \pm 0.06$
HACS	$681.93^{b} \pm 1.95$	$8.80^{d} \pm 0.03$	$12.70^{d} \pm 0.36$
CS	$520.67^{c} \pm 0.15$	$11.50^{c} \pm 0.00$	$15.60^{c} \pm 0.00$
WCS	$492.17^{d} \pm 0.91$	$12.20^{b} \pm 0.00$	$17.93^{b} \pm 0.06$
WS	$407.80^{e} \pm 0.50$	$14.70^{a} \pm 0.00$	$22.13^{a} \pm 0.06$

Notes: Results are expressed as means ± SD, the superscripts following each figure in the same column indicate significant difference ($p < 0.05, n = 3$). WCS: waxy corn starch; CS: corn starch; WS: wheat starch; RS: rice starch; HACS: high amylose corn starch; $d_{3,2}$: surface-area-based mean diameter; $d_{4,3}$: volume-based mean diameter.

7.3.3 Complexing index measurement

The complexing index (CI) value of wheat starch-milk fatty acid complexes increased when the concentrations of fatty acids increased, as shown in Figure 7-2. Normal wheat, corn, and rice starches all complexed with milk fatty acids and stearic acid, in agreement with previous studies [381, 384, 385]. High amylose corn starch displayed remarkable CI value, which is in line with a previous study on amylose-stearic acid [386]. Whereas waxy corn starch exhibited a comparable CI value to corn starch, demonstrating the complexing ability of pure amylopectin, consistent with a previous report on waxy wheat starch-fatty acid complexes [385].

It needs three helix pitches (6 – 8 glucose per pitch, 180.156 g/mol) to entrap a milk fatty acid (225 g/mol) molecule [387]. Assuming 6 – 8 glucose molecules per helical pitch form an inclusion complex with a single milk fatty acid, thus 225 g of lipids and 3.243 – 4.324 kg starch are able to make a mole of milk fatty acid-starch complexes, equal to a starch-lipids ratio (w/w) of 14.4 – 19.2, or 5.2 – 6.9% of lipid (starch base) for complete complexation. For full complexation, fatty acids (FA) to starch mass ratio would be: $CI(100\%) = MW_{FA}/(3 \times 7 \times 180.156) = 5.95\%$. Therefore, the CI of milk fatty acid-starch complexes can be calculated with the equation of w $CI(100\%) = 16.82x$, where x is the mass ratio of milk fatty acids to starch, and MW_{FA} is the average molecular weight of milk fatty acids. The equation could be used to estimate the CI value of wheat starch-milk fatty acids, as illustrated in the midpoint estimate line of Figure 7-2a. Per this equation, it takes 17 starch units (w/w, lipid based) or 5.95% milk fatty acids (w/w, starch based) to make 100% CI. For instance, the

estimated CI value at 5% fatty acids was 84%, approximately close the measured value (72.58 ± 0.28%, Figure 7-2a).

The CI value of starch-fatty acid complexes is dependent on both the constituent species and the manufacturing complexation process. For example, the CI value exhibit an inverse relationship to the chain length of fatty acids-shorter fatty acids can more effectively disperse in gelatinized gels, thereby facilitating the interactions of starch with fatty acids and enhancing complexing indexes [378]. However, the equation can still be used to estimate the necessary amount (usually 5-10%) of lipids needed to form complete complexes with starch. For instance, 5% stearic acid has been used to complex with debranched starch (*ca.* 70% amylose) [176, 386], whereas the mass ratios of stearic acid-brown lentil starch and corn oil-corn starch were 10% and 5%, respectively [374, 381]. In this study, three mass ratios of 4%, 12%, and 20% were designed to represent partly complexed, fully complexed, and fatty acid surplus.

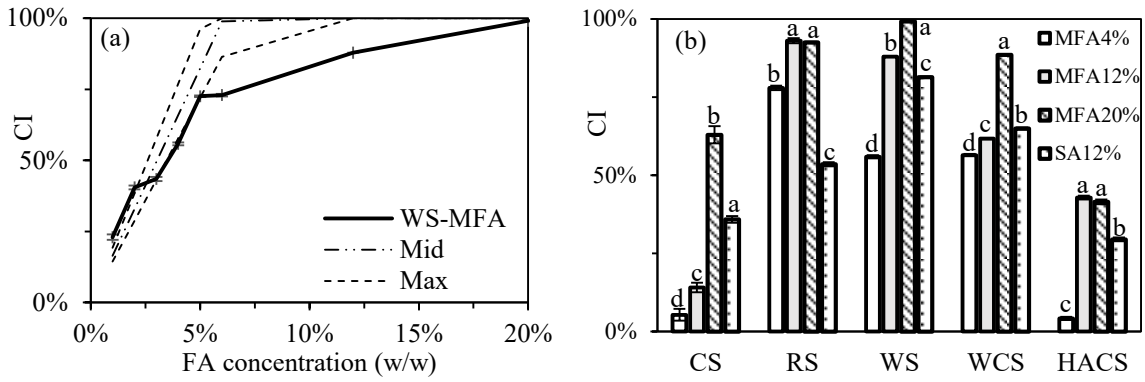


Figure 7-2: CI of starch-milk fatty acids.

Notes: The different superscripts on each bar of the same starch indicate significant difference ($p < 0.05, n = 3$); CS: corn starch; RS: rice starch; WS: wheat starch; WCS: waxy corn starch; HACS: high amylose corn starch; MFA: milk fatty acids; SA: stearic acids; MFA0 (control), MFA4, MFA12, and MFA20 represented that the milk fatty acid concentrations in starches were 0%, 4%, 12%, 20%, respectively; SA 12 (side control): stearic acids supplementation at 12% (w/w, starch based).

7.3.4 Rheological analysis and gel firmness

As illustrated in Table 7-3, the formation of milk fatty acid-amylose complexes resulted in a concomitant significant decrease in peak viscosities except for corn starch gels (rapid gelatinisation), most likely due to the lubrication effects of milk lipids. There were increases in the final and setback viscosities ($p < 0.05$), consistent with a previous report by Ai *et al.*

[276]. With respect to the mechanism of rheological change in corn starch, at the beginning, the granule swelling was restricted by the added milk lipids and the formed complexes, leading to the lower viscosity value than those of controls [388]. The increase in the final viscosities (FV) with the increase in percentage of milk lipids was related to the milk lipids re-solidification during the final, cooling stage of rapid viscosity analysis (RVA) standard-2 procedure [374].

In addition to pasting properties, the gel firmness decreased with the increase in milk fatty acid concentration, as shown in Table 7-3. As a consequence of stearic acid addition, the gel strength of SA12 samples became less than those of MFA0 (controls), similar to the trend with milk fatty acid addition (MFA4, 12 and 20). The complex-induced gel softening was more pronounced in wheat and corn gels than rice gels. As a consequence of lipid addition, the gels became softer than those of the controls, in agreement with a previous report on corn starch-palmitic acid (10%, w/w) complexes [276]. Furthermore, the gel firmness results were supported by previous reports on potato- and Bambara starch-linoleic acid, stearic acid, and lysophosphatidylcholine complexes [376] and on corn starch-stearic acid complexes [389]. The conformation of starch-fatty acid complexes (lamellar crystallites) reduces the water-binding and gel-forming ability of starch particles, thereby weakening the gel structure.

Table 7-3: Gel rheological characteristics and starch digestibility.

Samples	MFA0 (control)	MFA4	MFA12	MFA20	SA12
WS					
PV (cp)	$532^{a} \pm 13.5$	$429^{ab} \pm 75.5$	$233^{b} \pm 194.0$	$442^{ab} \pm 37.5$	$459^{ab} \pm 9.0$
FV (cp)	$3{,}911^{c} \pm 64.3$	$4{,}779^{b} \pm 302.4$	$6{,}435^{a} \pm 209.1$	$5{,}964^{a} \pm 451.5$	$6{,}086^{a} \pm 161.0$
PT (°C)	$81.10^{a} \pm 0.05$	$82.30^{a} \pm 1.10$	$81.55^{a} \pm 0.30$	$82.13^{a} \pm 0.63$	$81.38^{a} \pm 0.08$
GF (g)	$117.52^{a} \pm 0.06$	$66.64^{b} \pm 0.20$	$22.71^{d} \pm 0.13$	$21.61^{e} \pm 0.07$	$51.48^{c} \pm 0.41$
$T_{1/2}$ (min)	$39.2^{b} \pm 1.50$	$39.1^{b} \pm 4.15$	$55.4^{b} \pm 10.24$	$61.5^{ab} \pm 16.59$	$83.6^{a} \pm 12.07$
k ($\times 10^{-2}$, min^{-1})	$1.77^{a} \pm 0.07$	$1.79^{a} \pm 0.18$	$1.28^{ab} \pm 0.27$	$1.18^{b} \pm 0.28$	$0.84^{b} \pm 0.12$
R^2	0.96	0.99	0.98	0.96	0.93
AUC	$85.63^{a} \pm 2.70$	$73.44^{b} \pm 0.67$	$71.37^{b} \pm 4.59$	$71.44^{b} \pm 0.66$	$62.47^{c} \pm 2.54$
CI (%)	-	$56^{d} \pm 0.5$	$88^{b} \pm 0$	$99^{a} \pm 0.2$	$81^{c} \pm 0.2$
CS					
PV (cp)	$1{,}998^{c} \pm 8.1$	$2{,}321^{b} \pm 62.7$	$2{,}272^{ab} \pm 70.0$	$2{,}428^{a} \pm 17.7$	$1{,}985^{c} \pm 11.0$
FV (cp)	$3{,}324^{c} \pm 34.0$	$3{,}479^{c} \pm 95.4$	$4{,}094^{b} \pm 154.0$	$4{,}885^{a} \pm 385.4$	$4{,}563^{ab} \pm 177.4$
PT (°C)	$74^{a} \pm 0.2$	$73.50^{a} \pm 0.5$	$73.65^{a} \pm 0.26$	$73.67^{a} \pm 0.28$	$74.07^{a} \pm 0.25$
GF (g)	$176.82^{a} \pm 2.58$	$120.83^{b} \pm 0.29$	$87.56^{c} \pm 0.30$	$19.31^{e} \pm 0.18$	$28.75^{d} \pm 0.35$
$T_{1/2}$ (min)	$17.3^{b} \pm 0.27$	$25.0^{b} \pm 2.87$	$34.5^{b} \pm 9.27$	$70.0^{a} \pm 23.11$	$82.1^{a} \pm 8.15$
k ($\times 10^{-2}$, min^{-1})	$4.02^{a} \pm 0.06$	2.80 ± 0.33b	$2.12^{b} \pm 0.61$	$1.08^{c} \pm 0.41$	$0.85^{c} \pm 0.09$

R^2	0.96	0.97	0.97	0.95	0.98
AUC	$143.15^{a} \pm 2.04$	$123.72^{b} \pm 1.98$	$115.39^{b} \pm 2.12$	$95.15^{c} \pm 11.34$	$79.45^{d} \pm 1.41$
CI (%)	-	$5^{d} \pm 1.9$	$14^{c} \pm 1.5$	$63^{a} \pm 2.8$	$36^{b} \pm 1.1$
RS					
PV (cp)	$753^{a} \pm 7.1$	$302^{c} \pm 46.0$	$343^{c} \pm 5.1$	$332^{c} \pm 14.3$	$458^{b} \pm 2.5$
FV (cp)	$2,537^{d} \pm 15.5$	$3,684^{b} \pm 96.2$	$4,046^{a} \pm 64.1$	$4,138^{a} \pm 25.4$	$2,858^{c} \pm 50.5$
PT (℃)	74.75 ± 0.35	-	-	-	-
GF (g)	$18.99^{a} \pm 0.08$	$8.43^{bc} \pm 0.37$	$8.91^{b} \pm 0.24$	$6.11^{d} \pm 0.07$	$8.20^{c} \pm 0.17$
$T_{1/2}$ (min)	$19.35^{b} \pm 4.96$	$57.45^{a} \pm 1.36$	$60.18^{a} \pm 5.06$	$69.98^{a} \pm 9.46$	$63.00^{a} \pm 6.11$
k ($\times 10^{-2}$, min^{-1})	$3.78^{a} \pm 1.14$	$1.21^{b} \pm 0.03$	$1.16^{b} \pm 0.09$	$1.00^{b} \pm 0.15$	$1.11^{b} \pm 0.10$
R^2	0.96	0.94	0.95	0.94	0.94
AUC	$134.59^{a} \pm 4.16$	$92.74^{c} \pm 1.00$	$101.36^{b} \pm 7.75$	$108.59^{b} \pm 0.71$	$92.29^{c} \pm 2.92$
CI (%)	-	$80^{b} \pm 0.7$	$93^{a} \pm 0.8$	$93^{a} \pm 0.3$	$54^{c} \pm 0.7$
WCS					
PV (cp)	$2,666^{ab} \pm 4.7$	$2,757^{a} \pm 68.6$	$2,485^{cd} \pm 82.1$	$2,383^{d} \pm 24.3$	$2,549^{bc} \pm 22.5$
FV (cp)	$3,298^{d} \pm 139.1$	$3,072^{d} \pm 153.4$	$5,145^{b} \pm 242.8$	$5,778^{a} \pm 52.5$	$3,748^{c} \pm 153.2$
PT (℃)	$74.28^{a} \pm 0.73$	$73.53^{a} \pm 0.03$	$73.95^{a} \pm 0.00$	$74.65^{a} \pm 1.13$	$74.13^{a} \pm 0.23$
$T_{1/2}$ (min)	$28.4^{c} \pm 1.51$	$41.0^{c} \pm 2.45$	$55.8^{b} \pm 2.90$	$58.1^{b} \pm 11.05$	$80.6^{a} \pm 2.44$
k ($\times 10^{-2}$, min^{-1})	2.45 ± 0.13^{a}	1.69 ± 0.10^{b}	1.24 ± 0.07^{c}	1.22 ± 0.21^{c}	0.86 ± 0.03^{d}
R^2	0.92	0.94	0.92	0.92	0.95
AUC	126.93 ± 4.13^{a}	118.84 ± 2.10^{a}	109.55 ± 2.91^{b}	107.60 ± 2.73^{b}	80.01 ± 3.81^{c}
CI (%)	-	56 ± 0.3^{d}	62 ± 0.2^{c}	89 ± 0.2^{a}	65 ± 0.4^{b}
HACS					
$T_{1/2}$ (min)	94.1 ± 1.96^{a}	-	94.1 ± 1.96^{a}	60.5 ± 1.09^{b}	-
k ($\times 10^{-2}$, min^{-1})	0.74 ± 0.02^{b}	-	0.74 ± 0.02^{b}	1.15 ± 0.02^{a}	-
R^2	0.96	-	0.96	0.95	-
AUC	56.34 ± 0.82^{b}	-	60.78 ± 2.17^{b}	72.45 ± 4.12^{a}	-
CI (%)	-	4 ± 0.5^{c}	43 ± 0.6^{a}	42 ± 0.8^{a}	30 ± 0.6^{b}

Notes: Results are expressed as means ± SD, the different superscripts following each figure in the same row indicate significant difference ($p < 0.05, n = 3$). CS: corn starch; RS: rice starch; WS: wheat starch; WCS: waxy corn starch; HACS: high amylose corn starch; PV: peak viscosity; FV: final viscosity; PT: pasting temperature; GF: gel firmness; AUC: area under the curve of predictive reducing sugar; $T_{1/2}$: half digestion time (min); k: the reaction rate constant ($\times 10^{-2}$, min^{-1}); CI: complexing index; MFA0 (control), MFA4, MFA12, and MFA20 represented that the milk fatty acid concentrations in starches were 0%, 4%, 12%, 20%, respectively; SA 12 (side control): stearic acids supplementation at 12% (w/w, starch based).

7.3.5 Fourier-transform infrared spectroscopy

Fourier-transform infrared (FTIR) spectroscopic analysis has been used to quantify the complexed molecules of amylose-fatty acid by characteristic groups, such as carbonyl group [286]. For instance, the wavenumbers 3437, 2922, and 993 cm^{-1} correspond to groups of hydroxyl (OH), C-H bond, and ether C – O – C, respectively. As shown in Figure 7-3, the characteristic signals at 3437 and 993 cm^{-1} from wheat starch were observed in the spectra of mixture of wheat starch and milk fatty acids, and the peak at 2922 cm^{-1} from milk fatty acids also appeared in the spectra of mixture. However, the spectrum of the complex displayed two new peaks at 3398 and 1024 cm^{-1} (repositioning by 39 and 31 cm^{-1}, respectively) from wheat starch, due to the reordering of starch helices when fatty acids were complexed inside the cavities of starch helices.

This shift of the carbonyl peaks was also observed in a previous study on high amylose maize starch-fatty acid ester complexes [390]. The shift of carboxyl bond peaks in the spectrum of starch-oleic acid complexes was also observed previously [377]. In addition, another study has demonstrated that the peak at 1662 cm^{-1} of *p*-Aminobenzoic acid (PA) was relocated to 1672 cm^{-1} in the spectra of PA-linear amylose complexes [391], indicating the formation of inclusion complexes with starch. Further, it has been shown that the molecular state of salicylic acid analogues changed from a crystalline state to an amylose inclusion complex by breakage of the hydrogen bond between carboxyl groups, causing a band shift from 1679 cm^{-1} (carbonyl symmetric stretching) and 1531 cm^{-1} (nitro asymmetric stretching) to 1728 cm^{-1} and 1537 cm^{-1}, respectively [392].

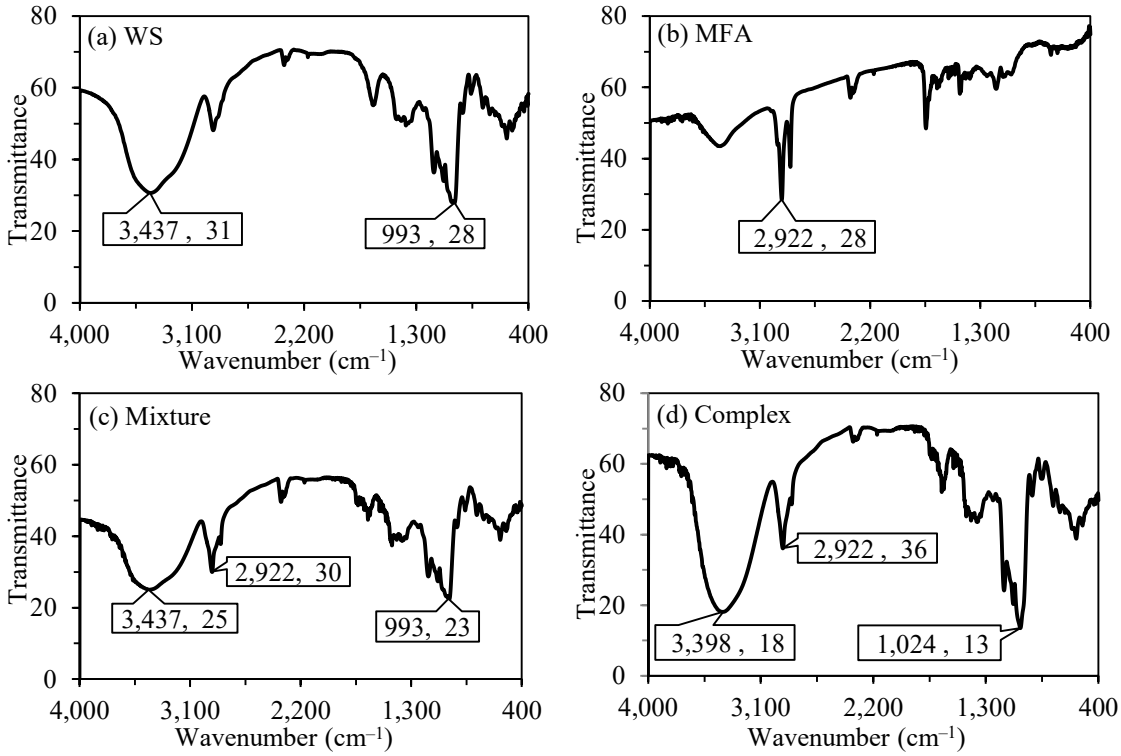


Figure 7-3: FTIR spectrums of WS-milk fatty acid complexes and their constituents.

Notes: a: FTIR spectrums of wheat starch (WS); b: milk fatty acids (MFA), c: mixture of milk fatty acids and wheat starch; d: milk fatty acid-wheat starch complex.

7.3.6 Thermography analysis

Previously, thermal analysis had been used to verify the formation of complexes [374]. As illustrated in Figure 7-4, the wheat starch spectra Figure 7-4b displayed an endothermic peak at 68.2˚C, indicating the pasting and gelatinisation of starch granules. Again, this peak was conserved in the DSC spectrum of milk fatty acid-wheat starch complexes, slightly shifting to 72.4˚C (Figure 7-4a). However, a new endothermic peak was observed at 109.6˚C, indicating the disassociation of complexes, in line with a previous report [374], where the unfolding temperatures of helical complexes were observed at 109.8 ± 2.12˚C and 107.1 ± 0.53˚C for brown lentil starch-stearic acid and brown lentil starch-palmitic acid complexes, respectively. In another study, the two transition endotherms for 3.75% (w/w) lauric acid-complexed wheat starch were at 63.8 ± 0.3˚C and 98.0 ± 0.5˚C, respectively, whereas only one gelatinization endothermic peak at *ca.* 62˚C was observed for native wheat starch [385]. The temperature differences of complex dissociation between Wang *et al*. [385] and the present observation on WS-MFA complexes are likely due to the affinity difference between the different constituents or the different types of fatty acids. The DSC thermogram of gelatinisation was for starch or starch-fatty acid mixture, and it was not applicable for fatty acids.

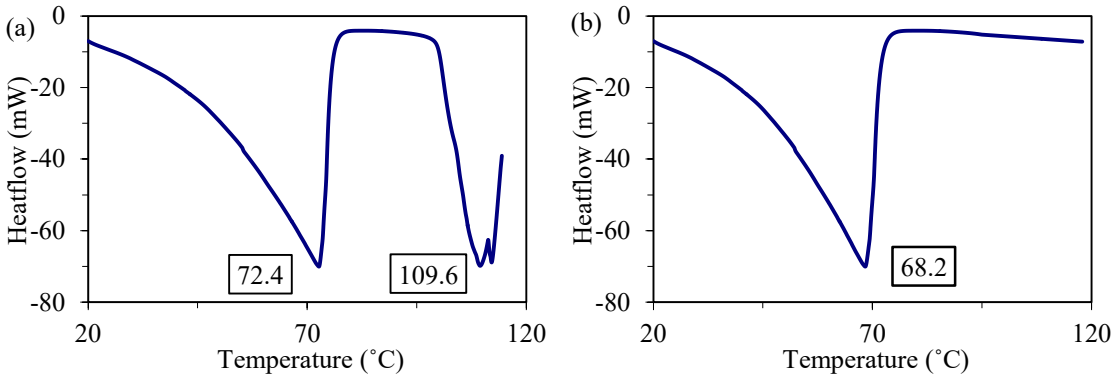


Figure 7-4: DSC thermogram of milk fatty acid-wheat starch complexes.

Notes: a: wheat starch-milk fatty acid complexes; b: wheat starch.

7.3.7 Starch hydrolysis kinetics

The starch hydrolysis followed by first-order kinetics ($R^2 = 0.95$), and the complexes of starch-milk fatty acids decreased the reaction rate constant of starches (wheat, corn, and rice), consistent with a previous report on rice flour digestion ($k = 2.04 \pm 0.11 \times 10^{-2}$ min^{-1}) [393]. The results were also in agreement with another report on fatty acid-starch complexes, where lotus seed starch had a reaction rate constant of $5.5 \pm 0.1 \times 10^{-2}$ min^{-1}, while the complexed starches had lower reaction rate constants than those of controls, ranging from $1.9 \pm 0.1 \times 10^{-2}$ min (C8), $2.2 \pm 0.1 \times 10^{-2}$ min^{-1} (C10), $2.1 \pm 0.1 \times 10^{-2}$ min^{-1} (C12), $2.9 \pm 0.2 \times 10^{-2}$ min^{-1} (C14), $5.3 \pm 0.2 \times 10^{-2}$ min^{-1} (C16), and $5.5 \pm 0.2 \times 10^{-2}$ min^{-1} (C18) [375]. One possible explanation for this decrease of reaction rate constants on complexation is that fatty acid-starch complexes restrict the swelling of starch granules and reduce the susceptibility of starch molecules to degradation by lipases, thereby attenuating the lipolysis reaction.

7.3.8 Reducing sugar release

As shown in Table 7-3 and Figure 7-5, with the increase of milk fatty acid addition, the CI value of MFA samples decreased, causing reduction of the AUC of reducing sugar release and the reaction rate constant (k) concomitantly except for HACS samples. The complexation with fatty acids decreased the AUCs of wheat, corn, rice, and waxy corn starch samples (MFA12) by 17%, 19%, 25%, and 14%, respectively. Whereas the AUCs of reducing sugar release of SA12 samples reduced 27%, 44%, 31%, and 37% for WS, CS, RS, and WCS groups, respectively, compared to those of controls. The reductions in the AUCs of reducing sugar release due to amylose-fatty acid complexation are consistent with previous reports on two stearic acid-complexed starches [176, 374, 375]. The reduction in the glycaemic load has been attributed to the attenuation of the hydrolysis velocity caused by amylose-fatty acid

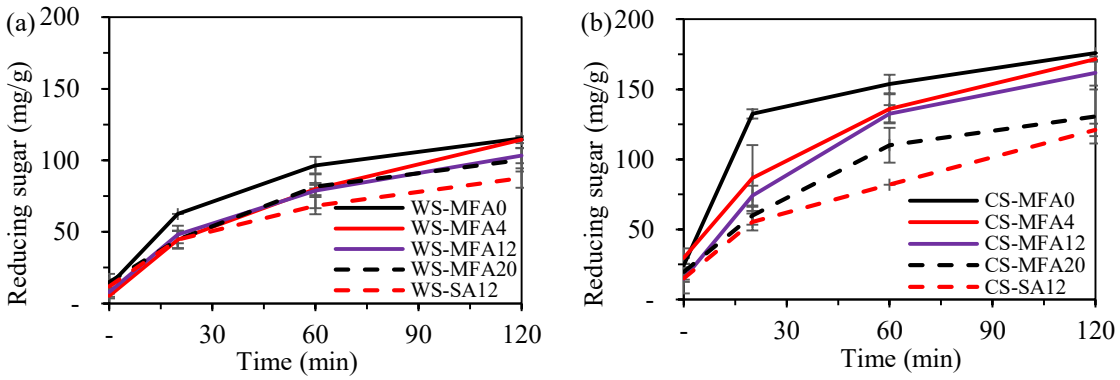
complexes [394]. In addition, the formation of complexes reduced the susceptibility of amylose to enzymatic hydrolysis *in vitro*. Wheat starch (WS), corn starch (CS), and rice starch (RS) showed different correlation numbers between AUC and CI. High correlation has been shown in WS and CS samples, c at –0.96 and –0.99 for WS and CS, respectively. Whereas, for RS, this number was as low as –0.89. It can be explained that starch digestibility is affected by both the quantity and quality of the single helix amylose-lipid complexes. For instance, although the complexation ability of capric acid and myristic acid complexed with rice starch was significantly higher than that of stearic acid, but the reduction of free sugar content was not significantly different. In addition, the CI value of rice starch and palmitic acid was relatively low, whereas a significant decrease in the digestibility of rice starch was observed in simulated gastrointestinal digestion [384].

The samples MPL12 (12% fatty acids, w/w) and MPL20 (20% fatty acids, w/w) displayed more effective resistance to amylase digestion in contrast to MPL4 (4% fatty acid, w/w) ($p < 0.05$), probably due to the increased complexing index. These results were consistent with a recent report on the digestibility of lotus starch-fatty acid complexes, where lotus starch-caprylic acid and starch-capric acid exhibited more resistance than those of palmitic- and stearic acid-complexed starches because the CI value in the former were higher than the latter [375].

High amylose corn starch (HACS) has a very compact physical structure, because its gelatinisation temperature (T_c = 109.3˚C) is high and its gel is not developed during the usual 95˚C cooking. Therefore, the hydrolysis velocity of HACS was very low, in conformity with a previous report on HACS [276]. HACS can form a strong gel, and be used to make biodegradable film and resistant starch. However, it has limited commercial application due to the challenge of producing it at normal cooking temperature conditions (60 – 95˚C, need 130˚C to fully and homogeneously gelatinize [395]). During heating at 95˚C (standard-2, 23 min including 7 min holding), the starch particles did not disperse sufficiently, thus confined starch molecules in the particle matrices yielded a low hydrolysis degree for HACS samples. When milk fatty acids and stearic acid were complexed with starch molecules, the crystalline starches became partly disrupted, resulting in unfolded starch granules. Therefore, the amylose binding site availability in starch increased, leading to a comparably higher digestibility of the lipid-treated HACS samples than the untreated controls. The enhancement of digestibility due to complexing was also observed in another report on lotus seed starch-fatty acid complexes, which were made by high pressure micro-fluidization [375].

Waxy corn starch also exhibited complexing capacity with milk fatty acids, thereby reducing the AUCs for reducing sugar release accordingly. These results were in line with previous study on waxy corn starch-lipid complexes [276, 396]. Compared to normal starch, waxy corn starch showed a remarkable hydrolysis resistance effect, which made it also an attractive source for the preparation of resistant starches.

The difference in the digestibility among milk fatty acid-complexed corn, rice, and wheat starches was significant ($p < 0.05$), as illustrated in the Figure 7-5, which can be explained by the variable ratios of amylose to amylopectin, granule size, retrogradation of amylose, and the degree of crystallinity [374]. The AUC value for the reducing sugar release from corn starch and wheat starch complexes were 143.15 ± 2.04 and 85.63 ± 2.70, corresponding to half-digestion constants of 17.3 ± 0.27 min and 39.2 ± 1.50 min, respectively, in line with a previous report on plasma glucose response of corn and wheat food [397]. In general, the digestibility decreased with the increase of the CI value, but the reduction of the glycaemic index (GI) is not inversely proportional to CI. For instance, rice-palmitic acid complexes exhibited a low CI value of only 30.9%, but their GI value was comparable with that of rice-myristic acid complexes [384].



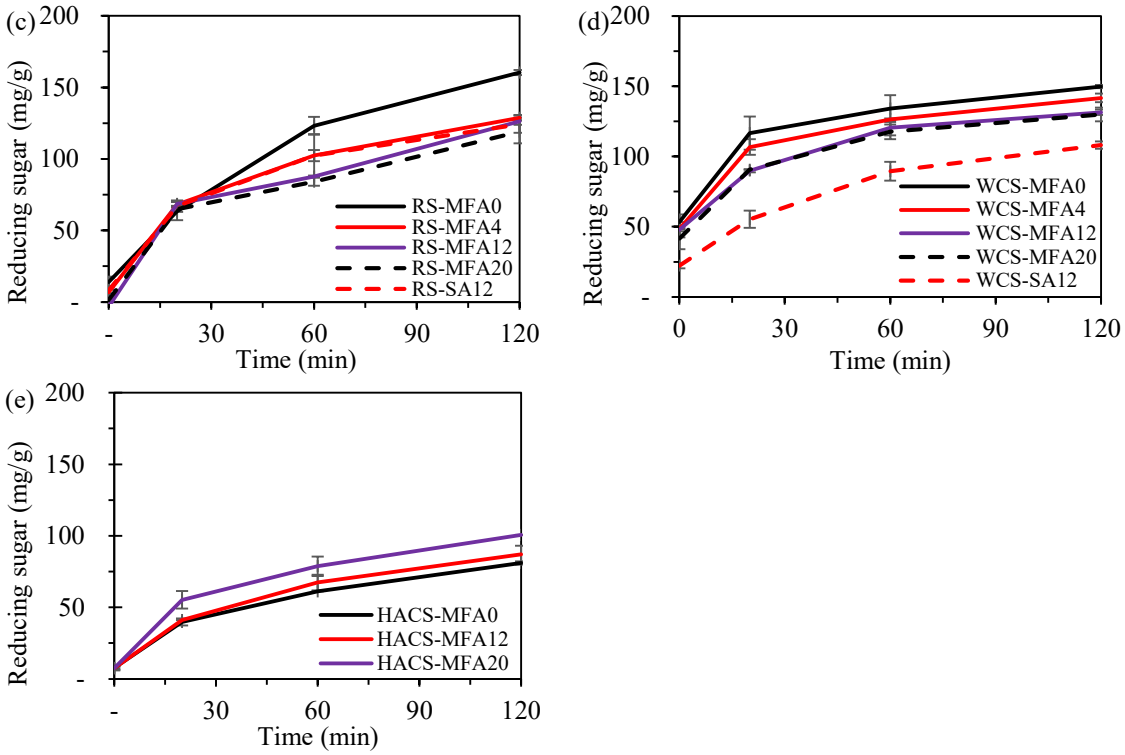


Figure 7-5: Predictive reducing sugar release of starch-milk fatty acid complexes.

Notes: Predictive reducing sugar release ($n = 3$) from: (a) waxy corn starch (WCS)-fatty acid complexes; (b) corn starch (CS)-fatty acid complexes; (c) wheat starch (WS)-fatty acid complexes; (d) high amylose corn starch (HACS)-fatty acid complexes; (e) rice starch (RS)-milk fatty acids (MFA); MFA0 (control), MFA4, MFA12, and MFA20 represented that the milk fatty acid concentrations in starches were 0%, 4%, 12%, 20%, respectively; SA 12 (side control): stearic acids supplementation at 12% (w/w, starch based).

7.4 Conclusion

To summarize, milk fat-derived fatty acids were successfully incorporated into rice, corn, and wheat starch gels, yielding various complexing indexes. The spectroscopy study confirmed the conformation of fatty acid-starch complexes. The conjugation with fatty acids led to weaker starch gels, with higher final viscosity and hydrolysis resistance than untreated controls. Compared to uncomplexed starch gels, the glycaemic indexes of the complexed starches were reduced by 19% in corn starch (CS), 17% in wheat starch (WS), and 25% in rice starch (RS), respectively. The half digestion time of milk fatty acid-complexed starches (MFA12, 12%, w/w) increased from 17.3 ± 0.27 min to 34.5 ± 9.27 min for corn starch, from 39.2 ± 1.50 min to 55.4 ± 10.24 min for wheat starch, and from 19.35 ± 4.96 min to 60.18 ± 5.06 min for rice starch. Thus these findings provide an approach to prepare cost-effective

resistant starches using milk lipids. These starch-milk fatty acid complexes can be further facilitated to produce resistant starchy foods for diabetic people.

Chapter 8: Milk lipid *in vitro* lipolysis kinetics in wheat, corn, and rice starch gels

Chapter 8 is published as:

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Abstract: Starch-based hydrogels or nano-emulsions have been used to regulate lipid digestibility. However, there have been a few reports on how starch gels affect milk lipid *in vitro* digestion. This study compared the pancreatic-lipase-catalysed digestibility of milk lipids in two matrices: milk fat dispersion and milk fat-loaded starch gels. Optical microscopy analysis suggested that lipid droplets were evenly distributed in starch gels. The peak viscosities of lipid-containing starch gels were higher than those of samples without filled-lipids, and whereas, final viscosities were reduced by lipid addition. Free fatty acid release measurement showed that milk fat embedded in three starch gels were two to three times more digestible than dispersed milk fat globules in aqueous phase. A multi-step hydrolysis reaction model was introduced to estimate lipolysis reaction rate. This study offers a feasible way to manipulate milk lipid digestibility by stabilization with starch gels.

Keywords: Milk fat lipolysis; multi-step reaction model; rheological characteristics; lipophilic encapsulation; particle size distribution.

8.1 Introduction

Milk fat accounts for one third of lipid intake in the Western diet [398]. The lipid digestibility has caused increasing concern in regard to cardiovascular disease and the nutritional value of milk products [399]. Burning fats instead of carbohydrates is crucial for endurance of marathon athletes [400], and lipid digestion and absorption may be regulated by emulsion technology [401] or by dietary components such as calcium, ethanol, and caffeine [402]. Whey protein isolate-chitosan stabilized emulsions demonstrated more stable structure than lipid emulsions, and thus could impede lipolysis through electrostatic attraction [401].

Lipid droplets may be stabilized in starch-based hydrogels, exhibiting different digestibility from lipid emulsion. For instance, Mun et al. [403] used mung bean and rice starches to make

filled hydrogels that accommodated β-carotene-loaded oil droplets, leading to significantly more release of free fatty acids in 2 h digestion than lipid emulsion. As a result of starch gelation, lipid droplets were trapped in starch gels, and therefore, their initial digestion rate was slowed down, compared to lipids in emulsions. Also, seaweed polysaccharides exhibited stabilising properties in oil-in-water emulsion system (O/W) [404]. In a report by Park et al. [288], β-carotene was solubilized in lipid droplets and further entrapped by starch hydrogels, altering both lipid digestibility and β-carotene bio-accessibility. Further, starch particles have been used as surface coating material to form Pickering emulsions, limiting lipase access to oil droplets [405]. In addition, curcumin in oil-in-water emulsion was stabilized by starch granule Pickering emulsions, yielding an encapsulation efficiency of 80% and elevated physical stability [406]. Also, starch particles were used to increase the barrier properties of Pickering emulsion to oil-water interface [407].

Although lipid droplet-filled starch gels and their functionalities have been studied elsewhere, there have been a few studies on how starch gels impact on lipolysis reaction kinetics so far. It has been hypothesized by Marefati et al. [406] that the inner side of starch granules towards the oil droplets may have a less easily digestible structure than external side of the granules. Similarly, the inner oil droplets could have a less easily digestible structure than the lipid emulsions without stabilized by starch gels because the outer layer of the double-layered structure must first be disrupted to initiate lipolysis. Therefore, this study aimed to stabilize milk fat with three kinds of starch gels (rice, corn and wheat), assess their pancreatic-lipase-catalysed digestibility, and predict the release of free fatty acids by a multiple reaction model.

8.2 Materials and methods

The materials and methods in the section of 8.2 can be referred back to the corresponding sections in Chapter 3, as illustrated in Table 8-1.

Table 8-1: Materials and methods for milk lipolysis.

Section 8.2	Materials and methods	As mentioned in the section of
8.2.1.	Materials	3.27
8.2.2.	Proximate nutritional analysis	3.28
8.2.3.	Preparation of recombined milk lipid dispersion and its turbidity analysis	3.29
8.2.4.	Preparation of starch gels to entrap milk fats and rheological analysis	3.30
8.2.5.	Light microscopy and confocal laser scanner microscopy (CLSM)	3.31

8.3 Results and discussion

8.3.1 Particle size analysis and microscopy

The average size ($d_{3,2}$) of milk fat globules in O/W emulsion was 16.90 ± 0.10 µm (MFG, control sample), consistent to microscopy image particle size in Figure 8-1d and Figure 8-1e, as analysed by a MasterSizer 3000 unit. The starch particle size ($d_{3,2}$) ranged from 5.46 to 14.70 µm in Table 8-2, as also observed by microscopy observation in Figure 8-1a– Figure 8-1c.

Lipid was distributed evenly in starch gels, as shown in the green spots in the CLSM images of RF-gel (3:1 w/w) in Figure 8-1d and WF-gel (3:1 w/w) in Figure 8-1e, and similar to an observation where non-dairy cream lipids were uniformly entrapped in starch gels [408]. As illustrated in CLSM image Figure 8-1e, the particle size ($d_{3,2}$) of the milk fat globules in wheat starch gel was 14.85 ± 2.80 µm by ImageJ analysis, comparable to that of control sample (MFG) and coarser than a previous optical microscopy observation [409]. Milk fat globules shown in Figure 8-1d – Figure 8-1e were out of shape, probably due to distortion from gel stress.

When starch dispersions were heated to 95˚C, and the hydrated starch particles swelled irreversibly. At the same time, amylose leached into aqueous phase, and the starch particles lost crystalline structures. Finally, the starch dispersions turned into viscoelastic gels [410, 411]. Starch particles appeared to be fully gelatinized during cooking and turned into irregular fragments, as illustrated in non-stained spots in Figure 8-1d and Figure 8-1e, consistent with a previous optical microscopy analysis on rice starch gelatinization [412]. During storage, amylose and amylopectin recrystallized successively [413].

Table 8-2: The specific surface area and particle size of starches.

Samples	Specific surface area (m^2/kg)	$d_{3,2}$ (µm)	$d_{4,3}$ (µm)
RS	1,100.67^a ± 3.06	5.46^c ± 0.02	8.45^c ± 0.06

CS	520.67^{b} ±0.15	11.50^{b} ± 0.00	15.60^{b} ± 0.00
WS	407.80^{c} ± 0.50	14.70^{a} ± 0.00	22.13^{a} ± 0.06

Notes: Results are expressed as means ± SD, the superscripts following figures of starch samples in the same column indicate significant difference among three starches ($p < 0.05$, $n = 3$). CS: corn starch; WS: wheat starch; RS: rice starch; $d_{3,2}$: surface-area-based mean diameter by laser diffraction; $d_{4,3}$: volume-based mean diameter by laser diffraction.

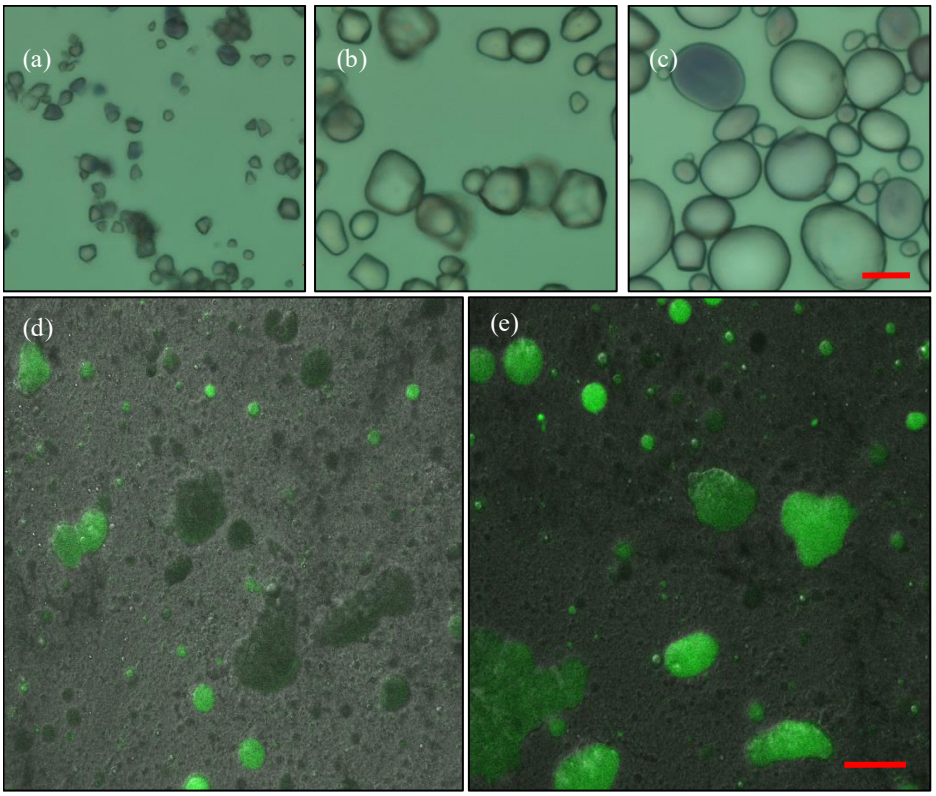

Figure 8-1: Light microscopy and CLSM images of milk fat-loaded starch gels.

Notes: Images in Figure 1(a – c) were viewed at 400-fold magnification, bright field optical microscopy for iodine-stained rice (a), corn (b) and wheat (c) starch. CLSM images in Figure 8-1(d – e) were observed at 400-fold magnification for RF- and WF-gel (3:1 w/w), respectively. Milk fat in green was stained by Sudan black, while starch was non-stained. Scale bars for images in Figure 8-1(a – c) and Figure 8-1(d – e) were 10 µm and 50 µm, respectively.

8.3.2 Starch gel characterisation

In contrast to side control samples (R-, C- or W-gels, 3:0 w/w), RF-, CF- and WF-gels (3:1 w/w) showed different viscosity in both the peak viscosity and the final viscosity, as depicted in Table 8-3. With the addition milk fat, the peak viscosity of RF- and CF-gels (3:1 w/w) were higher than those of side control gels (R-, C-gels), respectively, except for wheat starch, in

which the increased viscosity due to the addition of milk fat was counteracted by shortening effect (lubrication effect, reducing gel viscosity) of milk fat on wheat starch gel. Regarding to final viscosity, RF-gel (3:1 w/w) was found to be less viscous than R-gel without filling milk fat in. This is due to the reduced gelatinisation induced by shortening lipids (i.e. milk fat). In addition to the concentration of milk fat, the type of starch also had an impact on rheological properties of milk fat-filled starch gels.

During gelatinisation, the starch dispersions eventually turned into viscoelastic gels, in which amylose and water were bound by hydrogen bonding in three-dimension networks [414]. The viscosity of gels is associated to the degree of gelatinisation. Milk fat globules dispersed into starch gels and functioned as shortenings and fillers, separating and weakening the starch matrices [415].Therefore, the final viscosity of RF-gel (3:1 w/w) was less than that of fully gelatinized side-control (R-gel). Whereas, for CF- and WF-gels, no significant change in final viscosity was found between testing (3:1 w/w) and control (3:0) samples, due to synergistic effect of milk fat (i.e. both shortening effect and re-solidification). On one hand, milk fat restricted corn/wheat starch swelling and gelatinisation, thereby yielding less sticky gels [388]. On the other hand, milk fat re-crystalized and increased viscosity of gels at the cooling stage [374].

Table 8-3: Rheological properties of milk fat entrapped starch gels.

Samples	**Side-control 3:0**	**2:1 (w/w)**	**2.5:1 (w/w)**	**3:1 (w/w)**
RF-Gel PV (cp)	$753^{b} \pm 7.1$	$119^{d} \pm 8.2$	$498^{c} \pm 41.1$	$1{,}352^{a} \pm 53.8$
RF-Gel FV (cp)	$2{,}537^{a} \pm 15.5$	$893^{d} \pm 21.9$	$1{,}471^{c} \pm 8.2$	$2{,}068^{b} \pm 42.6$
CF-Gel PV (cp)	$1{,}998^{b} \pm 8.1$	$378^{c} \pm 47.1$	$1{,}755^{b} \pm 109.1$	$3{,}824^{a} \pm 157.6$
CF-Gel FV (cp)	$3{,}324^{a} \pm 34.0$	$1{,}120^{c} \pm 7.8$	$1{,}950b^{c} \pm 10.1$	$2{,}492^{ab} \pm 799.4$
WF-Gel PV(cp)	$532^{a} \pm 13.5$	$16^{c} \pm 1.2$	$108^{b} \pm 3.2$	$484^{a} \pm 4.4$
WF-Gel FV (cp)	$3{,}911^{a} \pm 64.3$	$726^{c} \pm 9.3$	$1{,}890^{b} \pm 21.5$	$3{,}801^{a} \pm 110.0$

Notes: Means that do not share a letter in the same row are significantly different ($p < 0.05$, $n = 3$). Tukey 95% simultaneous confidence intervals analysis was performed, with all pairwise comparison. RF-, CF- and WF-gel was denoted as the milk fat-load rice, corn and wheat starch gel, respectively. Side control (3:0) represented starch gel without milk fat. Starch gel 2, 2.5 and 3 were defined as the starch to lipid ratio (w/w) 2:1, 2.5:1 and 3:1, respectively. PV, peak viscosity; FV, final viscosity.

8.3.3 Hydrolysis degree of milk fat

As shown in Figure 8-2, the degree of hydrolysis of milk fat varied remarkably in the three kinds of starch-lipid ratio. Compared to control samples, milk fat in RF-, CF- and WF-gels exhibited 2 – 3-fold more hydrolysis degree in 2 h. The hydrolysis degree increased in the order of testing gels (2:1 w/w) < gels (2.5:1 w/w) < gels (3:1 w/w), in line to the order of starch gel viscosity. The difference in the milk lipid hydrolysis degree among the three kinds of starches was less obvious in contrast to the ratio of starch to lipids, because changing milk fat concentration has more impact on starch gel strength (*i.e.* more stabilisation effects) than changing the type of starch. In all three starch samples, each fatty acid release curve had a turning point, where starch gels were fully degraded by amylase catalysis, as illustrated in Figure 8-2a – Figure 8-2c.

Table 8-4: Hydrolysis degree of pancreatic-lipase-catalysed milk fat.

Samples	0:1 (Control)	2:1 (w/w)	2.5:1 (w/w)	3:1 (w/w)
RF-Gel	25.1^{c} ± 1.6%	$64.4^{b/A}$ ± 6.0%	$74.8^{ab/B}$ ± 6.0%	$78.3^{a/B}$ ± 0.0%
CF-Gel	25.1^{c} ± 1.6%	$13.9^{d/C}$ ± 0.0%	$48.8^{b/C}$ ± 6.0%	$78.3^{a/B}$ ± 0.0%
WF-Gel	25.1^{b} ± 1.6%	$27.8^{b/B}$ ± 3.0%	$93.7^{a/A}$ ± 6.0%	$97.1^{a/A}$ ± 0.0%
RF-Gel*	25.1^{c} ± 1.6%	$58.9^{b/A}$ ± 4.7%	$68.4^{ab/B}$ ± 5.5%	$71.6^{a/C}$ ± 0.0%
CF-Gel*	25.1^{c} ± 1.6%	$15.8^{d/C}$ ± 0.0%	$55.3^{b/C}$ ± 6.8%	$88.7^{a/B}$ ± 0.0%
WF-Gel*	25.1^{c} ± 1.6%	$22.9^{c/B}$ ± 4.9%	$77.2^{b/A}$ ± 4.9%	$79.9^{a/A}$ ± 0.0%

Notes: digestion in three starch gels in 2 h intestinal digestion; Results are expressed as means ± SD, the low-case superscripts following figures of starch samples in the same row indicate significant difference, and whereas the capital letter superscripts following means of data in the same column indicate significant difference among the different starches ($p < 0.05$, $n = 3$). RF-, CF- and WF-gel was denoted as the milk fat-load rice, corn and wheat starch gel, respectively. Control (0:1) sample represented milk fat emulsion without starch. Starch gel 2, 2.5 and 3 were defined as the starch to lipid ratio (w/w) 2:1, 2.5:1 and 3:1, respectively. *The 2-h intestinal hydrolysis degree of milk fat containing samples were normalized based on the same the milk fat globule area, which was assigned as 355.57 m²/kg.

8.3.4 Kinetic analysis of lipolysis in stepwise reaction

In addition to the experimental determination, a multi-step serial reaction model was used to simulate the fatty acid release [416]. The primary step was to free the encapsulated triacylglycerols (TAG^{*}) from starch gel matrices, which was dependent on starch hydrolysis kinetics (k_1). The freed TAG was then broken down with the lipase catalysis (k_2). The

hydrolysis reaction rate of starch was assumed to follow by first-order kinetics ($n_1 = 1$) [410, 417]. The lipolysis reaction rate of TAG was constant (zero order) at the beginning since enzyme concentration was much higher than that of lipidic substrates. Whereas, with the proceeding of lipolysis, the kinetics were first-order ($n_2 = 1$).

$$TAG^* \xrightarrow{k_1(n_1=1)} TAG \xrightarrow{k_2(n_2=1)Aqueous} 2FA + MAG \tag{8-1}$$

$$r_{TAG^*} = -k_1 C_{TAG^*} = \frac{dC_{TAG^*}}{dt} \tag{8-2}$$

$$r_{TAG} = k_1 C_{TAG^*} - k_2 C_{TAG} = \frac{dC_{TAG}}{dt} \tag{8-3}$$

$$r_{FA} = k_2 C_{TAG} = \frac{dC_{FA}}{dt} \tag{8-4}$$

$$C_{TAG^*} = C_{TAG_0^*} \exp(-k_1 t) \tag{8-5}$$

In which, C_{FA} and r_{FA} were the fatty acid concentration and free fatty acid release rate, respectively. C_{FA} can be obtained by overall mass balance of TAG.

$$C_{FA} = 2\big(k_2(1 - \exp(-k_1 t)) - k_1(1 - \exp(-k_2 t))\big)\, C_{TAG_0^*}/(k_2 - k_1) \tag{8-6}$$

The hydrolysis degree x of milk fat can be calculated from Eq. 8-7, where $t \to \infty$, then $x = 1$.

$$x = \big(k_2(1 - \exp(-k_1 t)) - k_1(1 - \exp(-k_2 t))\big)/(k_2 - k_1) \tag{8-7}$$

As illustrated in Figure 8-2, both the reaction rate and hydrolysis degree of milk fat in starch gels were higher than that of control sample, except for RF-gel (2:1 w/w), in which gelatinisation was insufficient due to the shortening effect of milk fat. For example, the control lipid emulsion showed a hydrolysis degree of 25.1 ± 1.6%, while the lipolysis degree was 78.3 ± 0.0% in the RF-gel (3:1 w/w). In regard to kinetics, the lipolysis reaction rate constant k_2 for lipid emulsion was 0.016 min^{-1}, and whereas, those (k_1) for starch hydrolysis were 1.77×10^{-2} (WS), 4.02×10^{-2} (CS) and 3.78×10^{-2} (RS) min^{-1}. The difference in k_1 may be related to such factors as amylose-amylopectin composition and starch-lipid complexes [276], swelling power and aqueous solubility [418], endogenous flour lipid [419], starch particle size [420], cooking degree and starch gelatinisation [421]. For instance, wheat starch hydrolysed slower than rice and corn starch, and whereas, rice starch exhibited comparable digestibility to corn starch, consistent to the starch digestibility of control samples in a previous report [419]. This can be explained by the mechanism of milk fat hydrolysis. Lipolysis, a bio-interfacial process, is mainly governed by the binding of the lipase-colipase-bile salt complex on the

surface of lipid droplets. Hence, by engineering oil-water interfaces, both the displacement by bile salts and the adsorption of lipase onto lipidic substrates can be modulated [422]. In addition to interfacial properties, other factors impact the lipolysis velocity and degree, including the emulsion structure [423] and stability [424], physical state (solid or liquid [425]), the extent of lipid emulsification [426], lipid sources [427] and molecular composition such as chain length [428, 429] and saturation [430].

Gastric pH and salts can alter both the size and the structure of particles, which further affect the intestinal digestion. However, gastric digestion contributes to only a minor proportion (10 – 30%) of the total phospholipid hydrolysis [426, 431] and takes place at pH 1.5 – 3, which is incompatible with the present titration quantification since both gastric acid and free fatty acids consume titrant. Therefore, only intestinal digestion was surveyed in this study, as also commonly performed by recent reports on lipid digestion [309-311].

In the present study, through stabilization of lipid droplets in starch gels, milk lipid digestibility was improved. For instance, the lipolysis degree increased by *ca.* two times in RF- and CF-gels (3:1 w/w) and by *ca.* three times in WF-gel (3:1, w/w), compared to that of control sample. In terms of mechanism, milk lipids comprise mainly of the stearic acids, oleic acids, and palmitic acid fatty acids, which are easy to coalesce or flocculate from dispersion to solid state during 37˚C hydrolysis. During the digestion of milk fat, there have been observation of coalescence [432], disk-like aggregation of native milk fat observed by CLSM due to a decrease in the charge of the milk fat globule membrane [398], or lipid droplet destabilisation during gastrointestinal digestion [433]. Secondly, for milk fat-loaded starch gels, with disintegrating of the starch gels, the released milk fat was exposed to lipase gradually. Therefore, both starch and milk lipids became hydrolysed concomitantly. In addition, compared to control sample, the surface areas of milk fat globules in starch gels were improved, as they were less likely to aggregate. Further, lipase adsorption in starch gels may be enhanced as well. Therefore, the milk lipid digestibility was enhanced as a result of colloidal structural change, consistent with a previous report by Mun et al. [403], in which mung bean starch gel-stabilized lipids hydrolysed to a greater extent than the lipids without stabilisation by starch gel.

The free fatty acid release predicted with the above multiple reaction model was consistent with the measurement in the samples of RF- and CF-gels (3:1 w/w), except for WF-gel (Figure 8-2d). For the hydrolysis degree of RF- and CF-gels (3:1 w/w) in the first 30 min and at final point (2 h), there was insignificant ($p > 0.05$) difference between experimental data

and predicted value, and whereas, for other time slots, the prediction model lacked fitness. For WF-gel (3:1 w/w), the prediction was significant different from the measurement, probably due to the simplification of the stepwise model. However, it has been found that the degree of lipid hydrolysis x changed with both reaction rate constants k_1 and k_2 (Figure 8-2e), which provides a way to regulate lipolysis by modulating starch digestion rate, for example, changing starch digestibility (*i.e.* resistant, slow or rapid digestion starch), source (rice, corn or wheat), and starch-milk fat mass ratio.

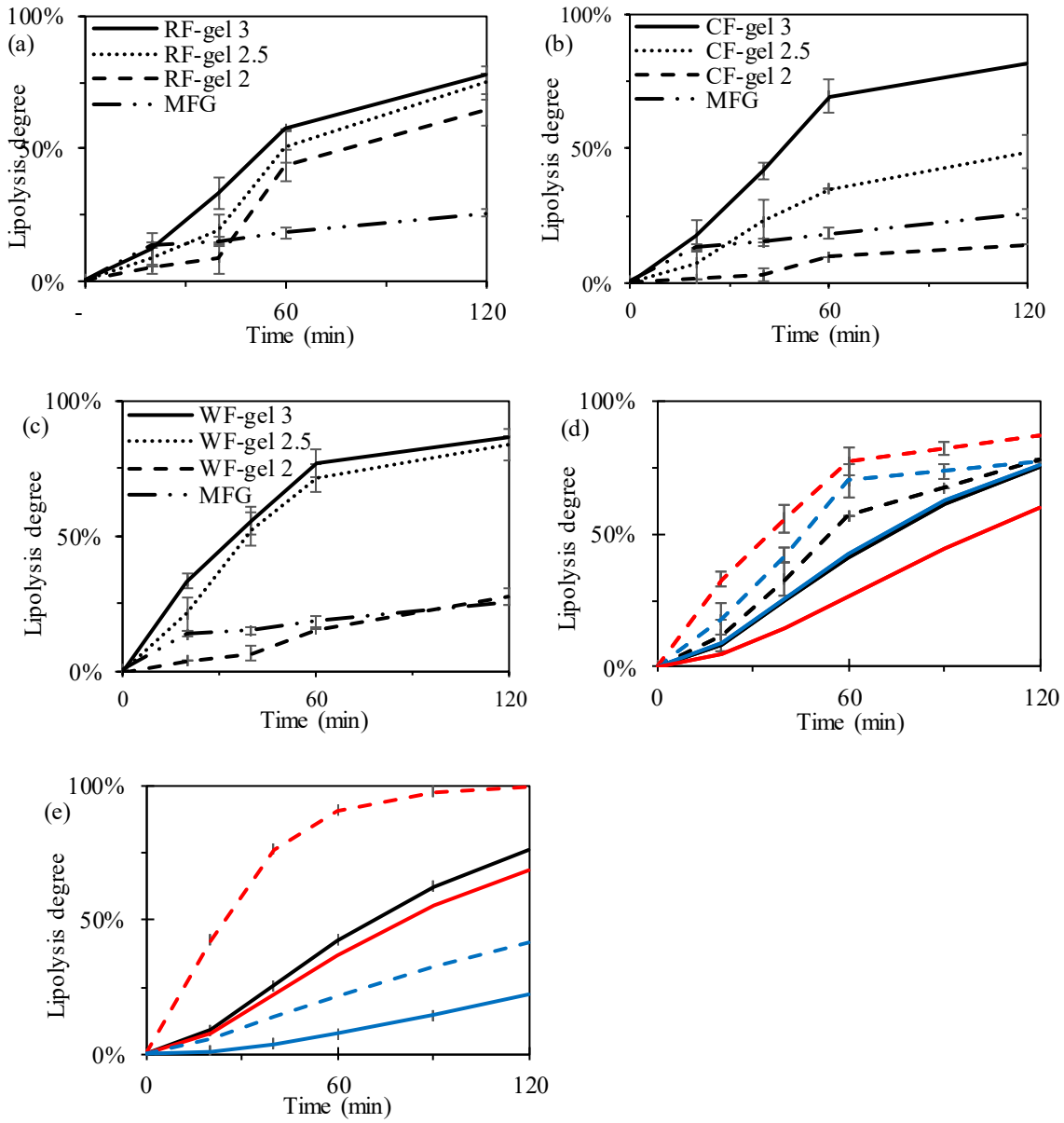

Figure 8-2: Pancreatic-lipase-catalysed lipolysis of milk fat in starch gel.

Pancreatic-lipase-catalysed lipolysis of milk fat in rice (a), corn (b) and wheat starch gel. Milk fat lipolysis degree by measurement and prediction (d) and by stepwise reaction model

simulation (e). Amylose-catalysed reaction constants: k_1; lipolysis reaction rate: k_2. RF-, CF- and WF-gel was denoted as the milk fat-load rice, corn and wheat starch gel, respectively. MFG represented milk fat globule emulsion (control). Starch gel 2, 2.5 and 3 were defined as the starch to lipid ratio (w/w) 2:1, 2.5:1 and 3:1, respectively. Dash lines of black (– – –), blue (– – –) and red (– – –) in Figure 8-2e represented the observed lipolysis degree of RF-, CF- and WF-gel, respectively. Solid lines of black (——), blue (——) and red (——) in Figure 8-2e represented the predicted lipolysis degree of RF-, CF- and WF-gel, respectively. Solid line in black (——) in Figure 8-2e represented the measured data for CF-gel (3:1 w/w), and whereas, Dash lines of blue (– – –) and red (– – –), solid lines of blue (——) and red (——) in Figure 8-2e represented the predicted lipolysis degree of CF-gel (3:1 w/w) at reaction rate constant of $3k_1{-}0.3k_2$, $3k_1{-}3k_2$, $0.3k_1{-}0.3k_2$, and $0.3k_1{-}3k_2$, respectively.

8.3.5 Particle size and structural change during intestinal digestion

As illustrated in Figure 8-3a and Figure 8-3c, the average particle size ($d_{3,2}$) of milk fat globules increased at the first 10 – 30 min, followed by a gradual decrease after one hour. The milk fat globule fluctuation in digestion fluid was also record by another lipolysis study [434]. Apart from particle size change, their distribution patterns also varied during digestion, with broader distribution span (Figure 8-3b and d), where the smaller particles signalled the disintegration of milk fat globules, and whereas, the bigger particles indicated aggregations. After half an hour, new peaks emerged in pancreatic lipase-catalysed milk fat dispersion in Figure 8-3b, reflecting the aggregation of milk fat globules. The lipid aggregates were also observed by Mun et al [403, 435], likely due to the change of pH and or ion strength. This may also explain the delayed digestion of milk fat emulsion, compared to the milk fat stabilized in starch gels, where hydrolysis of starch gel was synchronous with that of milk fat.

During the first 30 min, the inner droplets coalesced, leading to the increase of particle size and the population of large particles. As reflected by the PSD analysis in Figure 8-3d, the aggregation was detected at the early stage, followed by coalescence in the middle and clear emulsion at the end. This morphologic change was consistent with the microscopy analysis in Figure 8-3e – Figure 8-3g, as also observed similarly by optical microscopy [309, 417] and CSLM analysis [436, 437]. The ImageJ statistics showed that the milk fat in CF-gel (3:1 w/w) started with approximately 3.97 ± 1.39 μm, and changed to 2.59 ± 0.29 μm at 40 min and 2.29 ± 0.83 μm at the end.

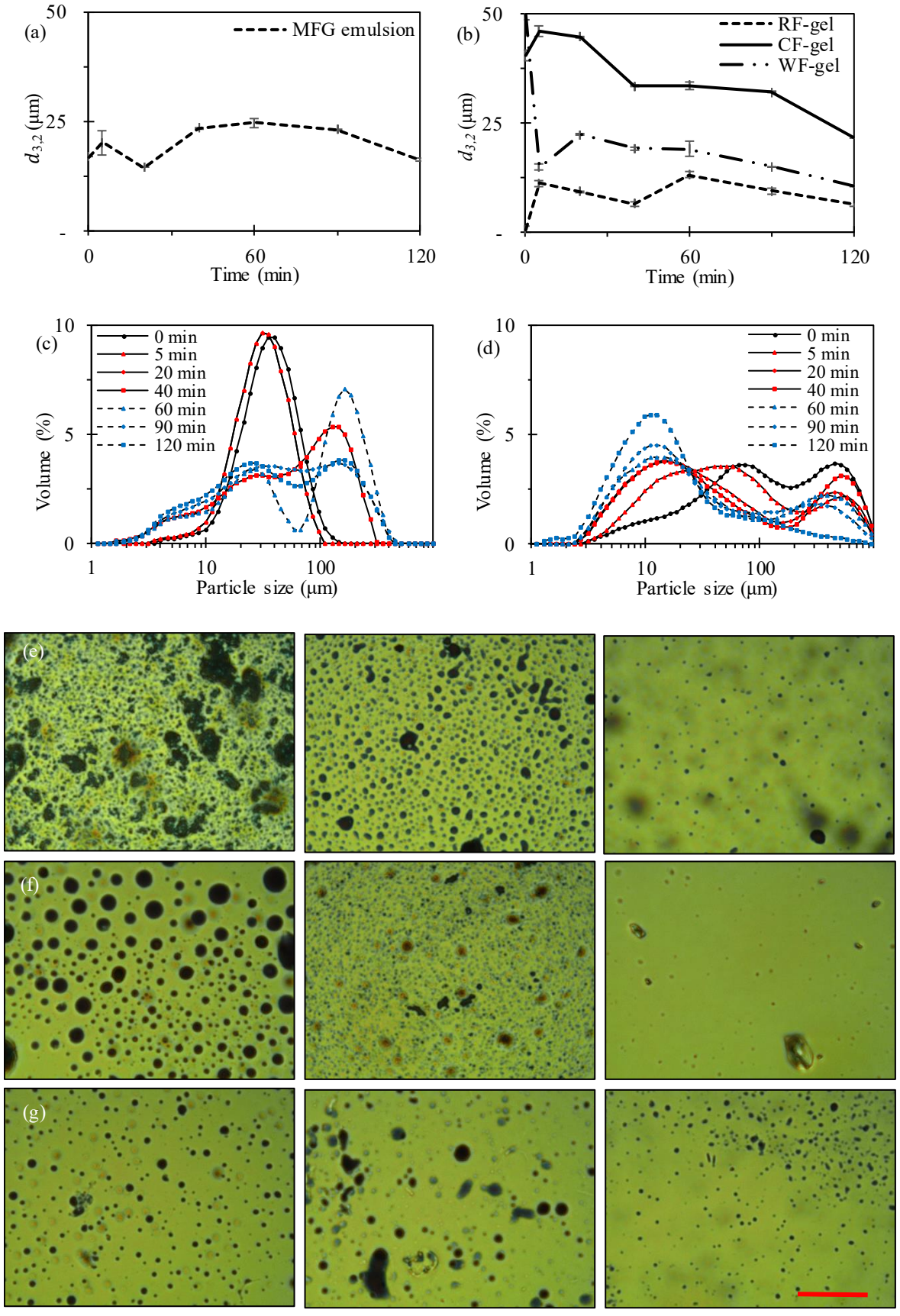

Figure 8-3: Structural change of MFGs during pancreatic-lipase-catalysed digestion.

Notes: Milk fat particle size ($d_{3,2}$, surface-area-based mean diameter) change during digestion was for MFG control (a) and milk fat containing starch gels (b), respectively. Milk fat particle size distribution was illustrated in MFG control (c) and CF-gel (3:1 w/w) (d), respectively. RF-, CF-, WF-gel were denoted for milk fat containing corn, rice and wheat starch gel (3:1 w/w), respectively. Morphology change of milk fat in 0, 40 and 120 min (from left to right), as stained by Sudan black, was illustrated for RF- (e), CF- (f) and WF-gel (g) (3:1 w/w), respectively. Scale bar for (e – g) was 20 μm.

8.3.6 **Implementation of barrier properties of hetero emulsions**

Lipophilic compounds such as curcumin, carotene, and α-tocopherol are unstable in aqueous solution but can be solubilized in a lipid dispersion. Emulsion-based food matrices have been designed as vehicles of bioactive compounds in foods and biopharmaceutical industries. For instance, β-carotene-laden lipids have been incorporated into hydrogels to improve its bio-accessibility, since β-carotene has poor aqueous solubility and chemical stability [288]. Also, starch granule Pickering emulsions have been used to deliver lipophilic curcumin, augmenting encapsulation stability [406]. In the present study, starch particle-stabilized lipid emulsions may also be utilized to carry fat-soluble compounds, and the hydrocolloid-based encapsulation can be produced by freeze drying (*i.e*. lyophilisation) or spray drying [438]. The bioavailability of many lipophilic compounds (*e.g*. polyphenolic, carotenoids, vitamin A, D, E and K) entrapped in lipid droplets, is dependent upon lipid digestibility, which is related to colloidal structure. For example, a starch nano-emulsion was used to deliver phenolic compounds from date palm pit [439]. Similarly, mixed emulsions can be used to protect functional food ingredients [401]. Additionally, the release rate of encapsulation content is proportional to that of carrier lipids [440].

8.4 Conclusion

Milk fat globules were filled into starch gels to regulate lipolysis kinetics in the present study. The milk fat-loaded starch gels generally enhanced the process of lipolysis by avoiding the coalescence of lipid dispersion, creating more surface area, and promoting surface activity for lipase binding to substrates. The lipolysis kinetics may be predicted by a multistep reaction model. In addition, the rheological characteristics of starch gels were measured to assess their impacts on fatty acids release. Milk fat lipolysis degree increased in the order of testing gels (2:1 w/w) < gels (2.5:1 w/w) < gels (3:1 w/w), in line to the order of starch gel viscosity. In general, milk fat stabilized in starch gels was two to three times more digestible than milk fat

without gel stabilization. With the disintegration of starch gels, milk fat was released from the gels concomitantly, followed by subsequent lipolysis, leading to increases of both reaction rate and hydrolysis degree of milk fat, especially for those milk fat-loaded starch gels (3:1 w/w). Hence, an energy food recipe (starch to milk fats 3:1, w/w) is recommended for athletes or sport foods. The results obtained in this study provide useful means of preparing new lipid matrices to encapsulate lipophilic nutraceuticals or to increase milk lipid digestibility in energy foods.

Chapter 9: Manufacturing Milk Phospholipid-Enriched Dairy Ingredients

Chapter 9 is published as:

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Abstract: Milk phospholipids (MPLs) have been used as ingredients for food fortification in products such as bakery products, yogurt, and infant milk formulas because of their technical and nutritional functionalities. Starting from either buttermilk or beta serum as the original source, this study assessed four typical extraction processes and have found that the life-cycle carbon footprints (CFs) of MPLs were 87.40, 170.59, 159.07, and 101.05 kg CO2/kg MPLs for membrane separation process, supercritical fluid extraction (SFE) by CO2 and dimethyl ether (DME), SFE by DME, and organic solvent extraction, respectively. Thus far, membrane separation remains the most efficient way to concentrate MPLs, yielding almost full recovery rate and 11.1 – 20.0% purity. Both SFE and solvent extraction processes are effective to purify MPLs to high purity (76.8 – 88.0% w/w). This approach offered a new parameter to assess the process intensity and energy efficiency to manufacture MPLs in large-scale.

Keywords: Milk phospholipids; buttermilk; life-cycle assessment method; carbon footprint; supercritical fluid extraction; membrane separation

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9.1 Introduction

Milk phospholipids consist of a subclass of polar lipids—glycerophospholipids and sphingolipids [13]. Glycerophospholipids comprise of a glycerol moiety with two fatty acids esterified at positions of sn-1 and sn-2 and a hydroxyl group at sn-3 position, linked to a phosphate group and a polar moiety [13]. The molecular structure of the latter determines the types of glycerophospholipids, namely phosphatidylcholine (PC), phosphatidylserine (PS),

phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidyl-glycerol (PG), and phosphatidic acid (PA) [14]. Sphingolipids consist of a sphingosine backbone (2-amino-4-octadecene-1,3-diol), connected to a fatty acid via an amide bond and a polar head. Sphingomyelin (SM), a prominent subclass of sphingolipids, has a phosphocholine residue [13]. In raw bovine milk, the diameters of milk fat globules (MFGs) are around 0.2 – 15 μm; these MFGs are enveloped by approximately 15 nm thick tri-layer MFG membrane (MFGM) [80, 441]. The composition of MFGM is 30 – 75% for polar lipids, and 25 – 70% for protein, respectively [45]. MPLs are within the MFGM constructing its backbone. MPLs represent 0.4 – 1% of the total milk lipids [54], which change with season, lactose stage, and feed [82].

MPLs have exhibited nutraceutical properties due to their profile of subclasses. MPLs contain high proportions of SM [442] and PS [443] (24 and 12%, respectively), subclasses which are virtually absent in other sources, such as soy (0 and 0.5%, respectively) and egg yolk lecithin (1.5 and 0%, respectively) [48]. PS, comprising 15% of the total phospholipid pool, is associated with resuming memory and releasing stress, and will be replaced by inactive cholesterol as brain ages [12, 444]. SM has been found to be effective in inhibiting colon tumours [445]. Also, MPLs have been implicated to mitigate the risks of Alzheimer's disease and repair cognitive ability [446], restore immunological defences, reduce the incidence of cardiovascular diseases [447, 448], and reduce cholesterol absorption and total liver lipids [449]. In addition, MPLs may narrow the gap between formula-fed and breastfed infants concerning neurodevelopment, infectious diseases, and cholesterol metabolism [450, 451]. Phospholipid-coated fats, *e.g.* human breast MFGs, will be more easily digested and absorbed, not only due to the size of MFGs, but also for the ratio of MFGM proteins to phospholipids [452]. Bovine MPL-enriched ingredients may be used to produce breast milk analogues. For instance, one formula recipe consists of subclasses by a weight-relative ratio of SM > PC > PE > PS > PI, with 21.1 – 29.7% SM and 10.2 – 13.3% PS (both based on total MPLs, similar to those of human breast milk (37.5% and 9%, respectively) [50]. Another infant formula comprises of 150 mg/L MPLs [453], mimicking that of breast milk (15 – 20 mg/dL milk [50], 0.3 – 1.0% of the total lipids [454]).

In addition to nutritional value and health benefits, MPLs have been technologically functional in food systems, for example, MPLs have been used in the preparation of liposomes [455], constructing vesicles of bioactive compounds [456], they are also food emulsifiers and surfactants, foaming agents, texture improvers for bakery goods, and may improve moisture retention for yogurt [457, 458].

Much research works and reviews regarding fractionation of MPLs have been published, for instance, fractionation from buttermilk (BM) and beta serum (BS) [457], isolating MFGs by washing and centrifugation [45], and membrane separation of polar lipids [442]. However, there is no standard large-scale manufacturing process adapted by the overall dairy food industry. This is due to many reasons. First, the native MFGM as the original source of MPLs is fragile. Shearing and turbulent fluid flow can cause damage to the MFGM [459]. These treatments are commonly involved in handling raw milk on farms, in transportation, in silos at manufacturing plants, and during cream separation. Damage to the MFGM may cause associated materials including MPLs to deplete from the native MFGs to the aqueous phase of milk. Therefore, more than half the portion of MPLs in raw milk are lost in skim milk [460, 461]. Second, uncertainties and variables are involved in the MPL fractionation processes. For example, cream washing for removing non-MFGM associated proteins may be performed before butter churning for increased yield or concentration of MPLs in the resulting BM or in the retentate of BM after tangential filtration. However, the cream washing procedure may interestingly only sometimes cause a significant change to the MPL composition in BM from unwashed cream [462, 463]. Although the detailed mechanism for explaining the phenomenon is not clear, it may relate to the stringency of different washing processes. Zheng's group revealed that different washing procedures induce various degrees of damage to MFGM. Therefore, washing may alter the composition of MPL in the fat phase of the washed cream [80, 464]. This study aims to assess different dairy streams rich in MPLs, to evaluate their extraction processes, compare their process intensity and efficiency, and to estimate their life-cycle carbon footprints (CFs) using ISO 14067 and greenhouse gas (GHG) protocols.

9.2 Milk Phospholipid Extraction from Dairy Products

9.2.1 Dairy By-Products Rich in Phospholipids

Commercial MPL products are usually derived from dairy products, such as BM [317], BS [442], acid cheese whey BM [465, 466], whey protein phospholipids concentrate (WPPC) [467] or whey BM [468]. Dairy streams in Table 9-1 comprise of 2.29 – 26.02% MPLs on a dry matter (DM) basis, varying with sources and processes.

BM is the product that remains after the removal of butter by churning cream and that may have been concentrated and/or dried as butter milk powder [469], as illustrated in Figure 9-1. Acid BM, a by-product of lactic butter, is made by churning cultured cream. Also, whey BM is produced by the churning of whey cream during cheese making [470]. WPPC is a by-

product during microfiltration (MF) of whey for manufacturing WPI. The permeate phase (milk fat discriminated phase) from this process goes forward for WPI manufacturing and the fat remaining phase (retentated phase) containing residual whey proteins is further concentrated for producing WPPC. A standard WPPC is comprised of more than 12% fat and 50% protein (DM), and less than 8% ash and 6% moisture according to American Dairy Products Institute (ADPI) 2015 [467].

BM, the serum phase resulted from the churning of cream, comprises of milk proteins and residual fat [317]. In terms of protein, lactose, ash and DM contends, BS and BM are very similar to those of cream products (Table 9-1) [18]. For instance, BM (FDC ID 454974) protein content is 3.33%, same as that of cream (FDC ID 495516). Though the fat content of BM is only one tenth of cream, MPLs of BM are 4 – 27-fold of raw milk, as shown in Table 9-1. The empirical equation $MPL = 0.0137 \times FC$ provides an estimation of the MPL content (g/L) of dairy product, where FC is the fat content of cream [52]. For instance, the estimated BM MPL content of anhydrous milk fat (AMF) from 80% cream, and of butter from 40% cream, is 1.1 and 0.55 g/kg, respectively. Whey BM, a by-product of whey butter, comprises of 6-fold the MPL content of raw milk in Table 9-1 [468].

BM and BS, the most abundant source of MPLs [471], have been underexplored or even treated as a waste stream [36]. For instance, a New Zealand-based dairy manufacturer used two-thirds of their BM for standardization, only one-tenth for BM powder (BMP), and their annual potential to produce MPL concentrate was 320 metric tons [36]. The annual BM output in Canada was 14.1 metric kilotons (18% of butter, 0.5% of bulk liquid [472]), compared to 20 kilotons in Belgium, 16 kilotons in Denmark, and 124.5 kilotons in German [473]. In 2013, approximately 5.2 million tons of BM was produced worldwide, similar to that of butter [317]. Worldwide, the annual BMP productivity was estimated as 410 kilotons (*ca.* 9.5% of butter), respectively, which has downstream applications in producing ice cream, baked goods' ingredients, low-fat Cheddar cheese, reduced-fat cheese, pizza cheese [470], or in replacement of skim milk powder for low-fat yogurt [474].

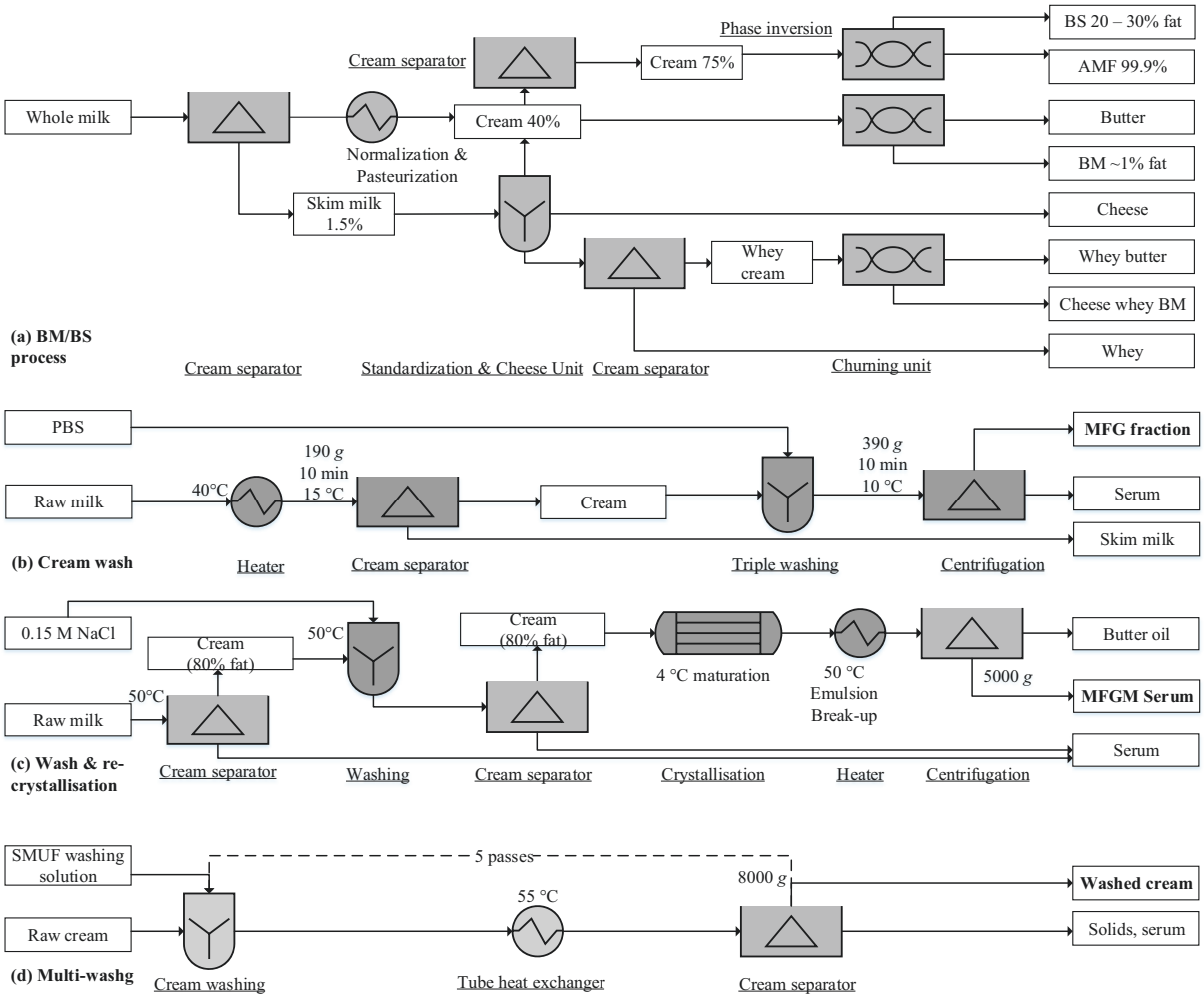

Figure 9-1: BM/BS production process and laboratory milk phospholipid processes.

Notes: (a) [457]; (b) cream wash [475]; (c) wash & recrystallization [2], (d) multi-wash [476]; BM, buttermilk; BS, beta serum; AMF, anhydrous milk fat;% indicates fat content; MFG, milk fat globule; MFGM, milk fat globule membrane.

Table 9-1: Dairy product composition.

Product	Total MPLs	DM MPLs	Fat MPLs	DM Protein	DM Fat	Total solid	DM Ash	Ref.
WPPC	1.60	7.92	29.10	65.00	27.00	20.20	7.92	[47]
WPPC	1.78	1.78	11.63	56.64 ± 0.05	24.23 ± 0.02	97.02	2.57 ± 0.02	[477]
WPPC	2.20	2.20	14.57	64.82 ± 0.12	18.71 ± 0.09	96.40	2.32 ± 0.01	[477]
WPPC	2.20	2.20	14.38	65.00 ± 0.06	18.46 ± 0.01	95.96	2.27 ± 0.03	[477]
BMP	1.30	1.30 ± 0.00	19.01	31.40 ± 0.57	6.84 ± 0.17	-	-	[317]
BM	0.14 ± 0.04	-	-	25.01 ± 0.76	12.22 ± 1.56	-	5.60 ± 0.16	[478]
BM	0.13 ± 0.00	1.43 ± 0.00	25.50	3.46 ± 0.05	0.51 ± 0.02	9.12 ± 0.17	-	[463]
BM	0.16 ± 0.02	1.78 ± 0.17	15.1 ± 0.5	32.44 ± 0.83	11.78 ± 0.53	9.02 ± 0.23	-	[54]
BS	0.97 ± 0.05	8.78 ± 0.41	38.6 ± 2.3	32.41 ± 1.01	22.71 ± 1.04	11.05 ± 0.43	-	[54]
BS	0.93 ± 0.07	8.42 ± 0.63	34.57	3.55 ± 0.11	2.69 ± 0.14	11.05 ± 0.40	-	[442]
BM	0.12 ± 0.01	1.36 ± 0.07	25.36	32.68 ± 0.93	4.87 ± 0.12	8.63 ± 0.26	-	[479]
BS	0.97 ± 0.17	8.8 ± 1.1	40 ± 7	33 ± 3	25 ± 8	11.0 + 0.8	-	[471]
BM	0.11 ± 0.01	1.2 ± 0.1	14 ± 5	33 ± 2	10 ± 5	8.7 ± 0.8	-	[471]
Whey BM	0.16 ± 0.01	2.01 ± 0.16	12.04 ± 0.8	24.89 ± 2.02	16.27 ± 2.06	8.05 ± 0.32	7.01 ± 0.47	[468]
BM454974	-	-	-	3.33**	3.33**	-	5*	[18]
BM336087	-	-	-	3.21**	3.31**	12.09	0.69/4.88*	[18]
BM171274	-	-	-	34.3	5.78	97.03-BMP	7.95	[18]
CM495516	-	-	-	3.33	36.67	-	3.33*	[18]
CM336519	-	-	-	2.84	36.08	42.19	2.74*	[18]

Notes: Unit, g/100 g; MPLs, milk phospholipids; BS, beta serum; BM, buttermilk; BMP, BM powder; WPPC, whey protein phospholipid concentration; CM, cream; DM, dry matter; *lactose; **wet basis; Ref., reference.

9.2.2 Commercialized Milk Phospholipid Products and Concentrate

Phosphoric 500/600/700 and Gangolac 600 (products manufactured by Fonterra) comprise of 34, 75, 62, and 15% MPLs, respectively, representing one source of highly-purified MPLs [31, 55]. Arla Foods Amba have implemented the phospholipid-rich dairy milk concentrated commodities for infant milk formulas and skin care. It has been claimed that Lacprodan® MFGM 10 supports physiological development of the infant gut and provides infants with similar health benefits to breast milk because of their similarities in fatty acid profile [56]. Arla dairy products PL 20/75 consists of 20% and 75% MPLs, respectively [57].

As illustrated in the patents in Table 9-2, both filtration and solvent extraction are validated processes to manufacture MPLs. Acetone and supercritical CO_2 are effective solvents for defatting processes. Tatua [58] and Synlait [59] have concentrated MPLs to 5 – 12.8% (w/w, DM basis). Lecico has implemented membrane separation to produce Lipamine M20 (20% purity) [60].

Table 9-2: Proprietary/patented manufacturing technologies of milk phospholipids.

Applicant	Input	Technology used	MPL content	Reference
Fonterra	BSP	SFE CO_2 defat, hi-pressure DME extract	65.7 – 75.5	[480]
Meggle	BSP	SFE CO_2 defat, hi-pressure ethanol extract	~98.5	[481]
Owen John	BSP	SFE CO_2 defat, ethanol co-solvent extract	PI/PS lost	[482]
Merchant & Gould	Cream	UF, DF	27.7 – 38.8	[483]
Marc Boone	BM	UF 5 – 20 kDa	~2.84	[484]
Land O'Lakes	BM, BS	UF, defat by SFE CO_2	>30	[485]
Morinaga	Whey BM	MF 0.2 µm, defat by SFE CO_2	~22	[486]
Snow Brand	-	Extract by acidic ethanol, defat	-	[487]
Enzymotec	-	Extract by ethanol & hexane, acetone defat	~24	[50]
Cargill	-	Extract by alcohol (C_1 – C_3), acetone defat	-	[488]
Svenska	BMP	Extract by ethanol & n-heptane, acetone defat	~70 SM	[489]

Notes: kg/100 kg products; MPLs, milk phospholipids; BM, buttermilk; BMP, BM powder; BS, beta serum; BSP, BS powder; MF/UF, micro/ultra-filtration; DME, dimethyl ether; PI, phosphatidylinositol; PS, phosphatidylserine; SM; sphingomyelin; SFE, supercritical fluid extraction.

9.2.3 Laboratory Extraction of Milk Phospholipids

Intact MFGM makes up 2 – 6% of the total mass of MFG [457]. However, MFGM represents 60 – 70% of the total milk phospholipids [5]. Raw bovine milk comprises of 0.2 – 0.4 g MPLs/kg, and raw milk is generally a laboratory source of MPLs [45, 46]. Intact MFGs can

be isolated with low-speed centrifugation. The cream layer from raw milk skimming can be washed with phosphate buffered saline (PBS; pH 6.8, 0.1M, 1:10, v/v) and centrifuged at 390 g for 10 min at 10°C. The final cream layer after three washes is the large MFG fraction [475]. Different from isolating intact MFGM, Sanches-Juanes et al [2] ruptured MFGM and recrystallized milk lipids, and starting from raw milk, they washed cream with 0.15 M NaCl solution and precipitated casein by centrifugation at 5000 g.

Cream washing is a step to remove casein from cream and to clarify MFGs [36]. After centrifugation, casein will precipitate, with lipid stratification at the top layer [476]. Also, calcium, naturally present in casein micelles, can form a complex between MFGM and the casein micelles through its binding to phospho-casein and phospholipids of MFGM, leaving impurities with MPLs [490]. In addition, washing causes severe loss of phospholipids, almost 60% per wash [463]. Hence, washing facilities for separating MPLs are costly and energy-intensive [36], thereby mainly for laboratory purpose [45, 476, 491].

For analysis purpose, MPL samples are usually prepared by solvent extraction. The Folch [62] and Bligh [63] methods use chloroform and methanol to dissolve lipids. Other lipophilic extraction formulas include the Mojonnier solvents [492], dichloromethane [61], and ammoniacal ethanolic solution of lipids with dimethyl ether and light petroleum in the Röse-Gottlieb extraction [51, 64]. Total lipid content in samples can be determined with a gravimetric assay, Gerber-van Gulik butyrometer, infrared spectroscopy according to an International Dairy Federation (IDF) method [51], or gas chromatography equipped with a flame ionization detector [65].

To determine MPLs and their subclasses, solid-phase extraction can fractionate polar lipids from non-polar lipids. Silica gel bonded cartridges or silica gel plates can be used for such purpose [35]. The obtained MPLs can be solvent dried by vacuum and stored at –20˚C before using [66]. In addition, chloroform and methanol are also valid elution solvents [67]. Total MPLs can be measured using the IDF molybdate assay [68], Fourier transform infrared spectroscopy [69] or fluorescence assay on cleaved choline group [70]. Both nuclear magnetic resonance of 31P and chromatography can quantify MPLs and their subclasses [71, 72]. High-performance liquid chromatography is more acceptable than thin layer chromatography, using such detectors as ultraviolet absorbance, evaporative light-scattering detector and mass spectroscopy [456].

9.3 Processes for Industrial Manufacturing of Milk Phospholipids

9.3.1 Solvent Extraction

Many kinds of polar solvents have been used to extract MPLs, such as ethanol and alkanes [50, 487]. To separate casein from MPLs, proteins can also be denatured thermally or in an acid solution (pH 4.6) [47, 51], the aggregated particles are subsequently filtrated. Regarding fractionation of MPL from WPPC, ethanol (70% v/v) at 60 – 80°C denatures proteins, after filtration the MPL concentration is *ca.* 45.8% in the filtrate in Figure 9-2a [47]. This notable method used no toxic solvent. However, the incompleteness of phospholipid recovery may be a concern [47].

Compared to 58.1% recovery by ethanol, tertiary amine $CyNMe_2$ (N,N-dimethylcycloexylamine) yielded a 99.96% recovery rate of MPLs. At various solvent-sample weight ratios, the lipid extraction was conducted at ambient temperature. The dissolved MPLs in the amine were released by bubbling CO_2 at atmospheric pressure, which converts CyNMe2 into the carbonate salt in Figure 9-2b. By injecting nitrogen and removing CO_2, the carbonate salt regenerated into the amine form for reuse (Figure 9-2b). Though the recovery rate for BM was as high as 99.96 ± 1.2%, the extraction rates for BS and concentrated BM were only 7.57 ± 0.59% and 77.27 ± 4.51%, respectively. In addition to input sensitivity, the amine may interact with dairy components and cause toxic consequences [493], and chemical facilities required may be incompatible in a dairy factory setting.

MPLs can be dissolved in ethanol and alkanes [50, 488, 489], and may not dissolve in acetone, ethyl acetate, and 2-pentanone [50, 488, 489]. Lipid BMP (100 g) dissolved in ethanolic hexane (1:4 v/v, 800 mL) under constant agitation at 45°C for 2 hours will produce an extract. The permeate of vacuum filtration can then be vacuum-dried at 1 kPa (Figure 9-2c). The residue (*ca.* 20 g) is then defatted twice with 120 mL acetone, and the resulting acetone insoluble (AI, *ca.* 7 g), mainly polar lipids, is in the final step vacuum dried again at 1 kPa [50]. However, acetone poses a degree of toxicity, as acetone residue in defatted MPLs may reach 5 – 10 ppm. Further, acetone can form a mesityl oxide via a condensation reaction, causing an off-flavour [494]. Hence, toxic residues in acetone-insoluble fractions need analysis and monitoring.

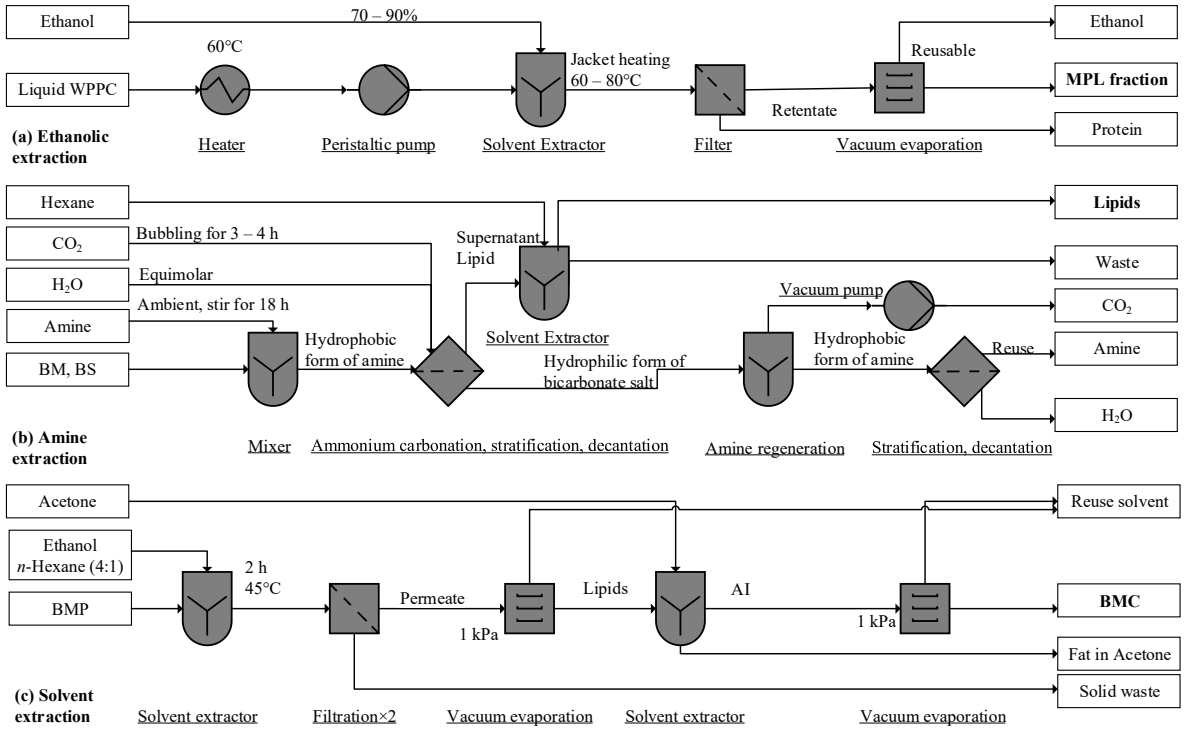

Figure 9-2: Process flow diagram of solvent extraction unit.

Notes: (a) adapted from [47]; (b) [493]; (c) [50]. BMC, buttermilk concentrate; MPL, milk phospholipid; AI, acetone insoluble; WPPC, whey protein phospholipid concentrate (liquid, reconstituted from powder).

9.3.2 Supercritical Fluid Extraction

Supercritical CO_2 and ethanol co-solvent can be used to extract MPLs effectively, yielding purities of 26.26% and 16.88% from WPPC and BMP extractions, respectively (Figure 9-3a). The SFE operation can be conducted at 50 – 60°C [495] and 350 – 550 Bar [477]. The SFE co-solvent (CO_2 and 20% ethanol) caused full extraction of polar lipids, including PE, PC, and SM. However, both PS (*i.e.* the vital compounds for cognitive function) and PI were not extracted, as reported separately [49, 482]. Therefore, the co-solvent method may be an invalid industrial process due to incompleteness of PS/PI recovery. In addition to co-solvents, near critical dimethyl ether (DME, 20 – 30% solubility) and supercritical CO_2 are able to dissolve polar and neutral lipids, respectively [480].

Supercritical fluid DME have been used to extract polar lipids, resulting in a yield of 69.1 – 77.8%. The SFE process shown in Figure 9-3b can accept both liquid (<45% lactose, DM basis) and powder input [480, 496]. This unit can work with CO_2 and DME in two-stages, extracting neutral and polar lipids, separately. In addition to its two-step operation, this unit can also operate a one-step extraction with DME. Near-critical DME dissolves both polar and

non-polar lipids in the SFE chamber. Through two-stage de-pressurization, lipids are separated from the protein fraction, whereas, vaporized DME will be compressed and condensed for reuse (Figure 9-3b). This method feathers non-toxicity, compact skid, feeding flexibility, and high content of MPLs (65.7 – 75.5 g MPLs per 100 g DM). However, MPL recovery rate (69.1 – 77.8%) needs further improvement.

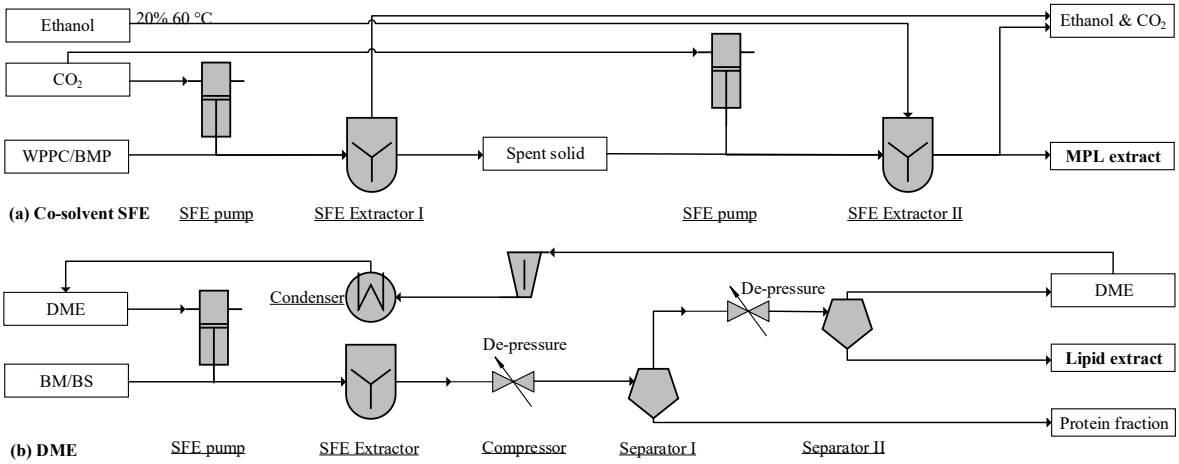

Figure 9-3. Process flow diagram of a supercritical fluid extraction unit.

Notes: (a) adapted from [477]; (b) [496]; WPPC, whey protein phospholipid concentrate; BS, beta serum; BM, buttermilk; BMP, BM powder; DME: dimethyl ether; SFE, supercritical fluid extraction.

9.3.3 Filtration to Enrich Milk Phospholipids

BM or BS is composed of milk fat, casein and whey protein, lactose and ash. The particle size (Figure 9-4c) ranges from 0.4 – 4 μm for MFGM fragment or phospholipid micelles [260], 0.02 – 0.3 μm for casein, 0.03 – 0.06 μm for whey protein, and 0.002 μm for lactose and ash, respectively [497]. The size of MFG is around 0.2 – 15 μm [441]. As illustrated in Figure 9-4a, the MF unit removes lactose and whey protein, and UF separates the smaller casein proteins from MPLs. Due to the size overlap of casein micelles and MPL particles, their separation is usually incomplete. Casein micelles disintegrate into peptides and amino acids in the proteolysis unit [52, 317], and hydrolysates enter into permeates during the subsequent UF operation [49, 52]. Alcalase (E.E. 3.4.21.62), a serine-type endoprotease with esterase activity, catalysed amino esters at pH 7.5 and 35 – 60˚C [49], while tryptic and peptic hydrolysis may be carried out at 42˚C for 2 – 16 h, at a pH of 7.7 and 2.0, respectively [52].

Membrane filtration is typical process to enrich BM (Figure 9-4b). Proteolytic treatment plus UF, as illustrated in Figure 9-4b, successfully differentiated MFGM from protein particles and yielded product purities of 14 ± 3.4% (DM) [52] and 11.05 ± 0.02 (DM) [49]. The combined process of proteolysis and membrane separation yielded a 100% recovery rate of MPLs in BM, as illustrated in Figure 9-4b. Considering membrane units exist in most dairy factories [43, 497], the process remains the most effective method to concentrate MPLs, requiring less investment than the other processes [498]. As illustrated in Figure 9-4b, this method [49] recovered more MPLs than the other processes.

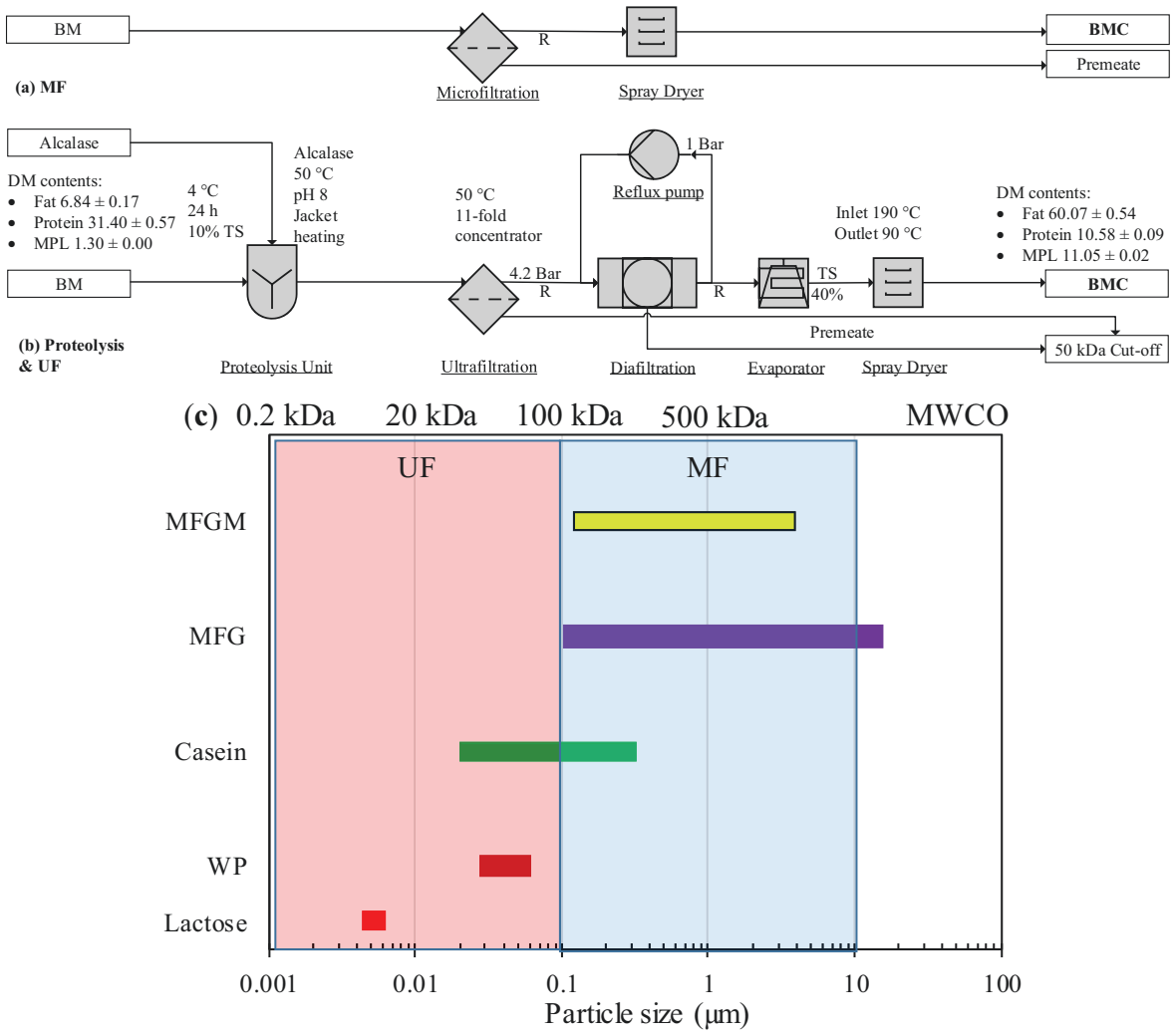

Figure 9-4. Filtration to enrich milk phospholipids.

Notes: (a) microfiltration (MF) [260]; (b) Ultrafiltration (UF) [49]; (c) particle size of dairy ingredients; MPLs, milk phospholipids; BM, buttermilk; BMC, BM concentrate; TS, total solid; R, retentate.

9.3.4 Typical Processes to Extract Milk Phospholipids

In brief, there are three options for large-scale manufacturing of MPLs, including solvent extraction [50, 489], SFE [480, 496], and proteolysis plus membrane concentration [49, 52, 64, 317]. Membrane concentration of MPLs have yielded 20% (w/w, DM basis) purity by Lecico [60] and Arla [48]. Tatua [58], Westland and Synlait [36] have extracted MPLs from BS powder (2.28%, w/w, DM basis), achieving approximately 12.8% (w/w, DM basis) purity by membrane filtration. The proteolysis and UF unit recovers MPLs completely [49, 64, 317] and cost-effectively [36]. This process is more efficient to produce MPL concentrate than SFE and solvent extraction. Whereas, SFE and solvent extractions are effective steps to manufacture high purity MPLs. Therefore, these three processes feature high recovery rate, facility availability, and food compatibility, representing current, best-available industrial practices (in Table 9-3).

Table 9-3. Process to purify MPLs and achieved purity.

Reference	Input	Technology used	Purity	Recovery (%)
[496]	BSP	SFE: CO_2 300 bar 40°C, DME	12.9 → 75.7 (5.9-fold)	69.1
[496]	BSP	SFE: DME 40 bar 50°C	12.9 → 66.8 (5.2-fold)	62.9
[477]	WPPC	SFE: 350 Bar CO_2, 20% ethanol, 60°C	2.2 → 26.3 (11.9-fold)	PS/PI lost
[477]	BMP	SFE: 550 Bar CO_2, 15% ethanol, 60°C	2.0 → 16.9 (8.6-fold)	PS/PI lost
[478]	BMC	SFE: CO_2 defat	2.2 → 7.8 (3.5-fold)	100
[478]	BMC	SFE: CO_2 defat	2.2 → 9.2 (4.2-fold)	100
[260]	BMC	SFE: CO_2 defat	9.6 → 19.7 (2.1-fold)	100
[468]	BMC	SFE: CO_2 defat	7.2 → 12.0 (1.7-fold)	100
[493]	BM	Solvent : BM (6:1 v/v) extraction	-	87.5
[493]	BM	Solvent : BM (12:1 v/v) extraction	-	99.9
[493]	BS	Solvent : BS (12:1 v/v) extraction	-	7.6
[52]	Whey BM	Proteolysis, UF/DF 300 kDa 40°C	0.3 → 8.6 (28.7-fold)	95 – 99
[52]	Whey BM	Proteolysis, UF/DF 300 kDa 40°C	0.4 → 11.4 (27.1-fold)	95 – 99
[52]	Whey BM	Proteolysis, UF/DF 300 kDa 40°C	0.5 → 14.0 (26.4-fold)	95 – 99
[49]	BMP	Proteolysis, UF/DF 50 kDa 50°C	1.3 → 11.1 (8.5-fold)	100
[317]	BMP	Proteolysis, UF/DF 50 kDa 50°C	0.8 → 6.2 (7.8-fold)	100
[499]	BM	MF 0.2 µm	1.5	67
[260]	BM	MF 0.8 µm	9.6	-
[463]	BM	MF/DF 0.5 µm 50°C	1.4 → 2.5 (1.8-fold)	88.8
[463]	BM	MF/DF 0.5 µm 50°C	1.4 → 4.1 (2.9-fold)	89.7
[478]	BMP	MF/DF 0.45 µm 9°C	1.2 → 2.2 (1.8-fold)	60.87
[478]	BMP	MF/DF 0.45 µm 9°C	1.5 → 2.2 (1.5-fold)	87.34
[478]	BMP	MF/DF 0.45 µm 9°C	0.5 → 0.9 (1.7-fold)	90.12
[478]	BMP	MF/DF 0.45 µm 9°C	0.3 → 0.7 (2.3-fold)	80.24
[465]	CWBM	UF 0.15 µm cellulose acetate	1.8 → 2.3 (1.3-fold)	41.9
[465]	CWBM	UF 0.15 µm cellulose acetate, TA	1.8 → 4.7 (2.7-fold)	98.4
[468]	CWBM	UF/DF 10 kDa	2.0→7.2 (3.6-fold)	-
[466]	CWBM	TA, wash at pH 7.25, UF/DF 55°C	2.0 → 10.7 (5.4-fold)	>90

Notes: unit: g MPLs/100 g dry product; BM, buttermilk; BS, beta serum; BMP, BM powder; BSP, BS powder; CWBM, cheese whey BM; WPPC, whey protein phospholipid concentrate;

BMC, BM concentrate; DME, dimethyl ether; SFE, supercritical fluid extraction; MF/UF/DF, micro/ultra/dia-filtration; TA, thermal aggregation.

9.4 Carbon Footprint

9.4.1 Life-cycle Accessment Method of Carbon Footprint

ISO 14040 life-cycle assessment (LCA) is an internationally accepted methodology to calculate a product's environmental footprint [500]. Life-cycle carbon footprints (CFs) of dairy products cover the direct emission from the dairy factory (scope 1), the energy carrier footprint for factory operations (natural gas, steam, power, nitrogen, and compressed air in scope 2), and the raw material, packaging and logistics in scope 3. In addition, life-cycle CF comprises of the emissions from the dairy farm (up-stream) and distribution centre (down-stream) [501]. The boundaries are set as shown in Figure 9-5a. The CFs of MPL products were reported as equivalent CO_2 emission for one kg of MPLs, according to the ISO 14067 reporting standards [502].

The CF of BM (baseline CF, 1.10 kg CO_2/kg BM powder) was cited directly from data in Canada derived from the ULICEES model [472], which follows by IPCC methodology [503], and covers emissions like methane [472], nitrous oxide [504], and carbon dioxide using the F4E2 model [505], and uses allocation matrix to partition six inventory flows (*i.e.* fuel, power, raw milk transportation, alkaline/acid, water and waste water) to 11 major dairy products [506].

Although MPL may be isolated from different dairy food streams, BM is used as the starting material to account for the CF of MPL-enriched products. The CF of MPLs is a sum of BM CF (as the baseline) and the CF of extracting MPL from BM at dairy factories, assuming that the BMP input is 100 kg (1.3%, w/w, DM basis). Both proteins and lipids in MPL concentrates acquire CFs according to a previous method [501]. However, only MPLs were considered as the target ingredients, without calculating that of protein in the MPL fractions.

The CF of BM concentrate (BMC) by membrane separation (MS) was calculated by the equation: $CF_{BMC} = CF_{BM} + CF_{MS}$, where CF_{BMc}, CF_{BM} and CF_{MS} were CF of BMC, BM and MS, respectively. The CF of MPL products by SFE or solvent extraction was calculated by the equation $CF_{MPLs} = CF_{BMC} + CF_{SFE}$ or$CF_{MPLs} = CF_{BMC} + CF_{Sol}$, where CF_{MPLs}, CF_{BMC}, CF_{SFE} and CF_{Sol} were CF for MPL product, BMC, SFE and solvent extraction process, respectively, as illustrated in Figure 9-5b.

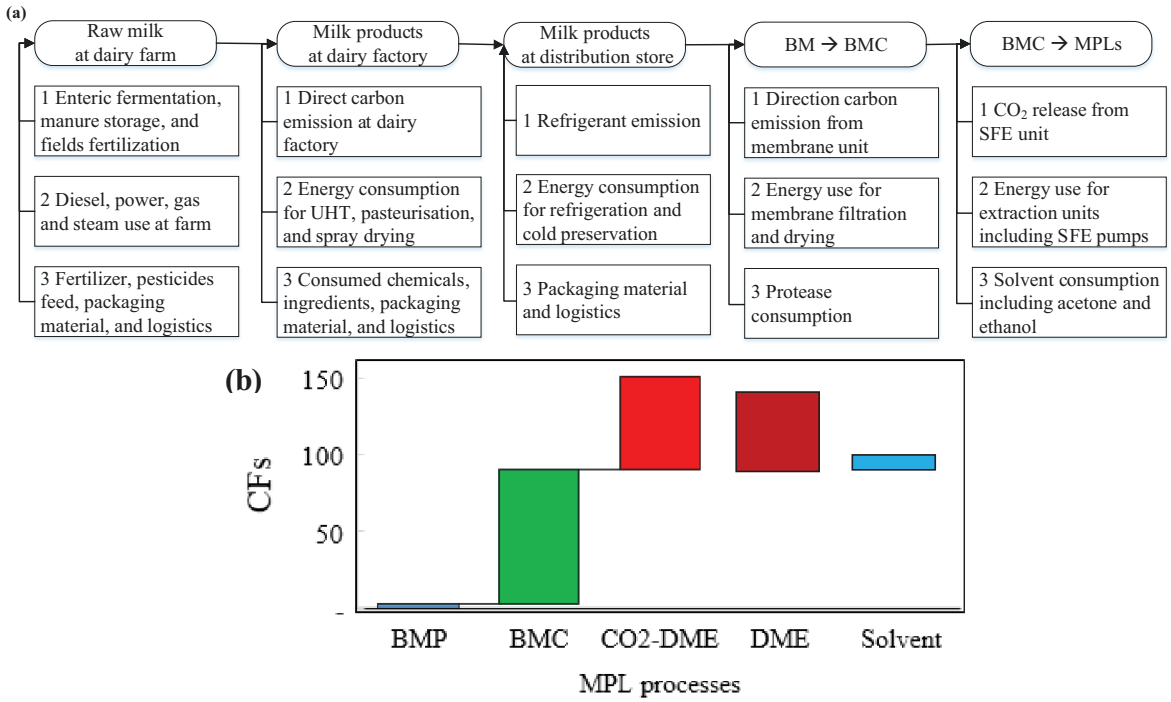

Figure 9-5. Boundary definition of the life-cycle carbon footprints of dairy products

Notes: (a) exemplary emissions from Scope 1 (direction emission), Scope 2 (energy carriers), and Scope 3 (raw material procured, packaging material and transportation); (b) cascade of CFs of BMP, BMC and MPLs by processes: "CO_2-DEM" (supercritical CO_2 and DME); "DME" (supercritical DME), and Solvent extraction (kg equivalent CO_2/products). Scopes of BM CFs: adapted from [472, 503-506]. MPLs, milk phospholipids; BM, buttermilk; BMC, BM concentrate; DME, dimethyl ether; SFE, supercritical fluid extraction; CFs, carbon footprints.

9.4.2 Carbon Footprint Estimation

In Table 9-4, four MPL enrichment processes are used as references for estimating and comparing total CFs. The membrane separation process was used to concentrate MPL from the original BM. The result product is BM concentrate (BMC). BMC may be further processed by either using SFE technique or by using the solvent extraction method. The further concentrate products are milk phospholipids (MPLs). The CF of "utility" consumed for three individual MPL enrichment methods was obtained by multiplying utility amount and CF conversion factor, which represents the amount of carbon emission for unit weight of utility. Normalized CF: $CF_{Normalized} = CF/C_{MPLs}$, where C_{MPLs} was MPL purity (g MPLs per 100 g product).

The normalized CF of product by membrane separation was as high as 87.4 kg CO_2/kg BMC, since the BMC comprised of only 11.05% MPLs. The CFs for products by SFE and solvent extraction were much higher than their baseline (CF_{BMC}), because of the intensive process during purification. As shown in Table 9-4, the CFs of fractions by SFE are 170.59 and 159.07 kg CO_2/kg MPLs for CO_2/DME co-extraction and DME extraction, respectively. CO_2/DME co-SFE exhibited comparably higher environmental impact to supercritical DME extraction, due to the direct emission from co-SFE. Solvent extraction demonstrated less environmental impact and a higher MPL recovery rate than SFE. However, the obtained products by solvent extraction are less food-compatible than SFE unit-extracted products.

Table 9-4. Normalized carbon footprints of milk phospholipids.

Process	Membrane	SFE (CO_2/DME)	SFE (DME)	Solvent extract	Unit
Reference	[49]	[480, 496]	[480, 496]	[50]	-
Input	BMP	BMC	BMC	BMC	-
Input amount	100.00	100.00	100.00	100.00	kg
Input purity	1.3	5.7	6.8	12.3	g/100 g DM
Product	BMC	MPLs	MPLs	MPLs	-
Product amount	11.76	5.13	6.56	13.98	kg
Product purity	11.05	76.80	66.80	88.00	g/100 g DM
MPL yield	100.00	69.10	67.40	100.00	%
Power	17.48	512.85	655.68	-	kWh
		CO_2 1,000.00		C_6/ethanol 552.00	kg
Material	Alcalase 0.03	DME 200.00	DME 200.00	Acetone 189.60	kg
Thermal energy	13.10	-	-	-	MJ
Power CF factor	0.1567	0.1567	0.1567	0.1567	kg CO_2/kWh
		CO_2 0.05	CO_2 0.05	C_6/ethanol 0.16	kg CO_2/kg
Material CF factor	5.00	DME 0.16	DME 0.16	Acetone 0.42	kg CO_2/kg
Thermal CF factor	0.06	-	-	-	kg CO_2/MJ
CF of power	2.74	80.36	102.74	-	kg CO_2
CF of material	0.16	82.00	32.00	167.95	kg CO_2
Thermal CF	0.72	-	-	-	kg CO_2
Utility CF	3.62	162.36	134.74	167.95	kg CO_2
BM/BMC baseline	110.00	498.17	594.31	1074.99	kg CO_2
Product CF	9.66	128.80	111.19	88.93	kg CO_2/kg
Normalized CF	87.40	170.59	159.07	101.05	kg CO_2/kg MPLs

Notes: BM, buttermilk; BMC, BM, concentrate; MPLs, milk phospholipids; C6, hexane; DME, dimethyl ether; SFE, supercritical fluid extraction; UF/DF, ultra/dia-filtration; CF, carbon footprint. Membrane filtration power consumption: 1.486 kWh/kg products [507]; Canada power CF factor: 0.1567 kg CO_2/kWh [508]; DME, reusable hexane and ethanol CF: 0.16 kg CO_2 /kg solvent; reused acetone CF: 0.42 kg CO_2/kg solvent [509]; DME CF 1.01 kg/kg; 84% reuse [510]; baseline of BM: 1.1 kg CO_2/kg BMP [472]; SFE CO_2 reuse 95% [511]; SFE CO_2/DME power cost estimation 100 kWh/kg extract [512].

MPLs of proteolysis and filtration process carry 87.40 kg equivalent CO_2/kg product, much higher than all the milk fat products (Table 9-5). With less CF than SFE and solvent extraction, membrane separation is the most efficient process among three accounted processes, in terms of process intensity, energy consumption and environmental impact. In addition, this process is compatible with most dairy factories, since membrane units have

already been commonly used facilities for whey protein concentration. Membrane separation is a necessary step to concentrate BM into BMC. Then BMC can be purified by SFE (DME). The relevant processes with significant MPL CF include membrane filtration, evaporation and spry drying, SFE and solvent recovery, offering opportunities to reduce the CF of the final products. For example, 0.1-μm polymeric spiral-wound MF membranes have been used to separate casein from milk, exhibiting higher energy efficiency at 0.024 (MF) and 0.015 (DF) kWh/kg permeate than that of graded permeability membrane (0.143 and 0.077 kWh/kg permeate for MF and DF, respectively [507]. Also, permeate flux, volume concentration ratio, transmembrane pressure, and temperature all had an impact on the energy efficiency of membrane UF, ranging from 0.26 – 0.33 kWh/kg retentate [513]. Another approach to reduce the environmental impact is to improve the purity of MPLs during filtration by differentiating the particle size of casein micelles (*i.e.* hydrolysis) from MFGM fragment and applying proper membrane filtration subsequently.

Table 9-5. Comparison of carbon footprint of MPLs in commercial dairy products.

Dairy products	CF	Scope 1	Scope 2	Scope 3	Country	Reference
Raw milk	1.10	-	-	-	Canada	[472]
Bulk liquid	1.00	0.870	0.065	0.065	Canada	[472]
Yogurt	1.50	1.083	0.252	0.165	Canada	[472]
Whole milk	1.12	0.843	0.173	0.104	China	[514]
Powder milk	10.10	-	-	-	Canada	[472]
Butter	7.30	-	-	-	Canada	[472]
BM	1.10	-	-	-	Canada	[472]
Cheese	12.40	-	-	-	Italy	[501]
Cheese	5.30	-	-	-	Canada	[472]
Cheese	8.80	-	-	-	Sweden	[515]
BM → BMC: UF/DF	87.40	-	-	-	-	[49]
BM → BMC → MPLs: SFE CO_2/DME	170.59	-	-	-	-	[496]
BM → BMC → MPLs: SFE DME	159.07	-	-	-	-	[496]
BM → BMC → MPLs: Solvent extract	101.05	-	-	-	-	[472]

Units: kg CO_2/kg product MPLs; milk phospholipids; BM, buttermilk; BMC, BM concentrate; DME, dimethyl ether; SFE, supercritical fluid extraction; UF/DF, ultra/dia-filtration; CFs, carbon footprints.

9.5 Conclusion

This paper identified three dairy streams for milk phospholipid (MPL) manufacture at an industrial scale: buttermilk, beta serum, whey protein phospholipid concentrate. The life-cycle CFs of MPLs were 87.40, 170.59, 159.07 and 101.05 kg CO_2 /kg MPLs for membrane separation process, CO_2/DME supercritical fluid extraction, SFE by DME, and organic solvent extraction, respectively. The extracted products comprised of 11.1, 76.8, 69.9, and 88.0% MPLs, with recovery rate at 100, 69.1, 67.4 and 100%, respectively. In conclusion, the combined process of proteolysis and UF is a preferred available process to enrich MPLs, with

full recovery of MPLs and the relatively low CF. SFE by dimethyl ether is the most effective method when needing high purity (*ca.* 66.8%) MPL products, at the cost of a high CF. This study provided insights into the best available industrial practices to extract MPLs and estimated their life-cycle CFs.

Chapter 10: Conclusion

10.1 Summary

In this thesis, two research chapters (Chapter 4 and Chapter 5) dealt with milk polar lipids and three (Chapter 6, Chapter 7, and Chapter 8) with milk fats. The related milk lipids were in the forms of either nutrient complexes (Chapter 4, Chapter 6, and Chapter 7) or within food matrices (Chapter 5 and Chapter 8), and the interactions between milk lipids and food ingredients (*e.g.* bioactive compounds or starches) were explored. This thesis demonstrated the effectiveness of milk phospholipid-based phytosomes as nutrient vesicles (Chapter 4), since a high complexing index of 98.52 ± 0.03% was obtained for phytosomes. Following vesicle properties of milk phospholipids, their digestibility and catalytic enzymes were compared with triacylglycerol. The results provided a basis to incorporate milk phospholipids into functional recipes, to enhance the physicochemical, nutritional and antioxidant activity of fortified foods (Chapter 5). In contrast to polar lipids, lipase-treated milk lipids were formulated into breads, strengthening dough and improving product textural properties (Chapter 6). Furthermore, milk lipid hydrolysates were applied to prepare amylose-fatty acid inclusion complexes, effectively yielding resistant starches (Chapter 7). In Chapter 8, starch gel-stabilized milk lipids were more digestible than those in dispersion, and therefore, this food matrix can be implicated to speed up lipid digestibility and increase endurance for athletes. The final research chapter (Chapter 9) dealt with the process development of extracting milk phospholipids from industrial streams including buttermilk and beta serum.

Overall, by complexing/interacting with antioxidants and starches, milk phospholipids/lipids have generated new nutritional functionalities in food systems, which can be utilized to deliver nutrients, regulate digestibility of starches/lipids, or produce fortified foods.

10.2 Conclusion

The findings on milk phospholipids study in this thesis work demonstrate three aspects: Firstly, milk phospholipids, although being minor constituents (*ca.* 1% of milk lipids) of tri-layered milk fat globule membrane, have exhibited vesicle ability to carry bioactive compounds with phytosomal complexes. The differences in encapsulation efficiency of two vesicles can be explained by their structural differences. In liposomes, bioactive compounds are trapped inside the bilayer or the core of milk phospholipids micelles, and whereas, phytosomes are integrated, stable structure (details as Section 4.4 in Chapter 4). Secondly, the

reaction rate of milk phospholipid lipolysis was faster than that of triacylglycerols, due to the differences in the hydrocolloid structures of the two emulsion matrices. Thirdly, although antioxidant activity of milk phospholipids was evidenced by *in vitro* assays, but their cellular antioxidant activity was very limited (details as Section 5.4 in Chapter 5).

The present study on milk lipids confirmed three major findings: Firstly, milk neutral lipids (*e.g.* triacylglycerol) in the core of milk fat globule, as predominant components of milk fat globules (*ca.* 98% milk lipids), could be conjugated with wheat starch, as texture improver and anti-staling agent for baked goods (details as Section 6.4 in Chapter 6). As a consequence of increased dough porosity, loaf volume and tenderness were improved in light of lipid lubrication and aeration mechanism during dough mixing. Further, the complexed amylose did not take part in gelatinisation, and therefore, upon cooling after baking, the complexed amylose will not recrystallize and participate in the staling of the bread crumb, thereby maintaining tenderness in storage. The staling process was delayed since the starch-lipid complexes hindered the amylopectin re-crystallisation and firming rate of crumb. In addition, the surface milk fats reduced the water holding capacity of starches due to their hydrophobicity and thus swelling and solubilisation of starches was also reduced. Inside the granules, the complexed amylose could be restricted from leaching-out during heating compared to free (non-complexed) amylose, thereby the amylose hydrolysis rate was delayed. Secondly, milk lipids could complex with corn, rice and wheat starches as resistant starch for diabetes. Besides, starch gel-stabilized milkfats demonstrate greater digestibility than milk lipids in dispersion, and therefore, they can be used to produce energy foods for athletes with optimal starch-to-milkfats ratio at 3:1 (details as Section 7.4 in Chapter 7). Thirdly, the starch particle-laden interface generally enhanced the process of lipolysis by avoiding the coalescence of lipid dispersion, creating more surface area, and promoting surface activity for lipase binding to substrates, thus speeding up the lipid digestibility compared to milk fat dispersion. The lipolysis kinetics can be predicted by a multistep reaction model (details as Section 8.4 in Chapter 8).

10.3 Technical relevance and significance of thesis findings

The significance to the field of milk phospholipids can be elaborated as follows: Firstly, to our knowledge, this is the first report of on preparation of milk phospholipid phytosomes and their functionalities. Phytosomes made out of milk phospholipids showed significantly higher encapsulation efficiency than liposomes, and therefore, they have potential functionalities in ingredient delivery systems and fortified foods, for instance, infant milk formulas (details in

Chapter 4). Secondly, the impact of polarity of milk lipids on lipolysis kinetics was examined, in addition to other important factors such as fatty acid chain length, saturation degree of fatty acids, hydrocolloid structure. Thirdly, milk phospholipids demonstrated *in vitro* antioxidant activity, which adds new insight on the design of milk phospholipid-fortified functional foods (details in Chapter 5).

The results on milk lipids studies provide evidence for new applicability of dairy fat products. Firstly, using milk fats as bread textural improver and anti-staling agent was explored in Chapter 6. This study provides a feasible way to improve bread quality and nutritional properties using milk lipids. Secondly, milk fatty acid-starch complexes provide an economical approach to produce low glycaemic response foods for diabetes using widely available food resources and an empirical equation was validated to predict the complexing indexes of milk fatty acid-starch complexing index (Chapter 7). Starch-stabilized milk lipids showed that showed greater lipolysis degree than milk fat emulsion, which was verified with a multiple reaction model. The findings of this study provide a feasible way to regulate lipolysis reaction rate with multiple reactions, and thus offer a basis to formulate milk fat-containing starchy, energy foods (Chapter 8).

For practical implementation of this thesis work, the results of this thesis can be used to develop milk lipid-based encapsulation systems (Chapter 4 and Chapter 8) in food and biopharmaceutical applications, lower starch digestibility for diabetes (Chapter 6 and Chapter 7), improve milk fat digestibility for prolonging of endurance of athletes (Chapter 8), and enhance the quality of baked goods (Chapter 6), as elaborated separately in below paragraphs. The complexation of milk phospholipids with antioxidants can be utilized as functional delivery systems, and whereas, the interaction and complexation of milk lipid-starch modulated digestibility of lipids/starches bring about applicability for making resistant starch and energy foods for diabetes and athletes, respectively.

10.4 Recommendations for further research directions

This thesis demonstrated functionalities of milk phospholipids/lipids in food systems. Milk phospholipid phytosomes showed higher encapsulation efficiency than liposomes, and whereas, milk phospholipids were found more digestible than triacylglycerol. In contrast, milk lipids were incorporated into bakery products, improving their physiochemical and nutritional properties. Furthermore, lipase-modified milk lipids can form complexes with rice,

corn and wheat starches, and produce resistant starch-based foods. Finally, starch-stabilized milk lipids showed greater lipolysis reaction rate than milk lipid dispersion.

In regards of future research interests for phospholipids, there have been emerging interests using them as improvers of cognitive performance or interventions of dementia such as Alzheimer disease, since milk polar lipids are more nutritionally-balanced than those from soya. Therefore, the neuro-functionalities of milk phospholipids may constitute the object of future studies.

On the other hand, further aspects regarding milk lipids and their interactions with protein and polyphenols, for instance, can be explored. The milk lipid-proteins interaction can be considered to understand their roles in modulating nutrient digestion and absorption. Additionally, milk lipids-polyphenol complexes can survive gastrointestinal digestion, increasing polyphenol bio-accessibility but decreasing fat absorption, for example. These complexes may also have beneficial effects on gut health. Regardless, interesting research questions for future research can be derived from the association of milk lipids with proteins or polyphenols.

Academy activities at a glance during thesis project.

A.1 Global food science student competition

Notes: Jiangnan University, Wuxi, China, Nov 11 – 14, 2018.

A.2 Summer university of ELLS

Notes: The Euro-league for Life Sciences (ELLS), Israel, June 22 – July 7, 2019.

A.3 Certificates from The Hebrew University of Jerusalem

THE INTERNATIONAL SCHOOL OF AGRICULTURAL SCIENCES
The Hebrew University of Jerusalem | The Robert H. Smith Faculty of Agriculture, Food and Environment

Euroleague FOR LIFE SCIENCES

ELLS Summer University 2019
Mediterranean Diet – from Genes to Health

Rehovot, Israel
23/6 – 4/7/2019

This is to certify that

<Huang Zhiguang >

Has actively and successfully participated in the 12 days Summer University organised by the Hebrew University on behalf of the Euro-league for Life Sciences (ELLS)[1] in co-operation with other EU partner institutions**".
The programme consisted of lectures, practical field and modelling work, and seminars.
The participants had to prove their knowledge and understanding of the topics in a final paper followed by presentation of their results and subsequent discussion.

Grade of presentation:

<87>

The national grading system of Israel is explained on the reverse side.

By successfully participating in this IP the student has been credited 7.5 ECTS.

Rehovot, Israel, July 15, 2019

Noa Schwarzwald
Course Coordinator

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